2019

Non-Canonical Functions Of The Bacterial Sos Response

Amanda Samuels
University of Pennsylvania, MANDIESAMUELS123@GMAIL.COM

Non-Canonical Functions Of The Bacterial Sos Response

Abstract
DNA damage is a pervasive environmental threat, as such, most bacteria encode a network of genes called the SOS response that is poised to combat genotoxic stress. In the absence of DNA damage, the SOS response is repressed by LexA, a repressor-protease. In the presence of DNA damage, LexA undergoes a self-cleavage reaction relieving repression of SOS-controlled effector genes that promote bacterial survival. However, depending on the bacterial species, the SOS response has an expanded role beyond DNA repair, regulating genes involved in mutagenesis, virulence, persistence, and inter-species competition. Despite a plethora of research describing the significant consequences of the SOS response, it remains unknown what physiologic environments induce and require the SOS response for bacterial survival. In Chapter 2, we utilize a commensal E. coli strain, MP1, and established that the SOS response is critical for sustained colonization of the murine gut. Significantly, in evaluating the origin of the genotoxic stress, we found that the SOS response was nonessential for successful colonization in the absence of the endogenous gut microbiome, suggesting that competing microbes might be the source of genotoxic stress. MP1 has an antimicrobial colicin under control of the SOS response, and colicins are hypothesized to function in inter-species competition. In Chapter 3, we therefore investigated the role of colicin in promoting successful colonization of MP1. We found that in the healthy murine gut, sustained colonization does not require colicin production, thus suggesting that MP1 existing in its natural niche does not face colicin-required microbial competition. This finding calls into question the importance of colicins in an unperturbed environment. Finally, with increased recognition of variance in SOS effectors across bacterial species, we posited that there may be a corresponding diversity in the regulatory LexA self-cleavage reaction. In Chapter 4, we systematically characterized LexA from phylogenetically-diverse bacterial species, uncovering a wide range of self-cleavage rates across bacterial species. Overall, this thesis describes non-canonical aspects of the SOS response by first exploring physiologic environments that require SOS activation, then investigating DNA repair-independent consequence of SOS induction, and finally probing the LexA self-cleavage reaction from multiple bacterial species.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Rahul M. Kohli

Second Advisor
Jay Zhu

Keywords
Colicin, Colonization, DNA damage, SOS response

Subject Categories
Microbiology

NON-CANONICAL FUNCTIONS OF THE BACTERIAL SOS RESPONSE

Amanda N. Samuels

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2019

Supervisor of Dissertation

_____

Rahul M. Kohli, M.D., Ph.D., Assistant Professor of Medicine

Graduate Group Chairperson

_____

Daniel S. Kessler, Ph.D., Associate Professor of Cell and Developmental Biology

Dissertation Committee

Jay Zhu, Ph.D., Associate Professor in Microbiology

Mark Goulian, Ph.D., Charles and William L. Day Distinguished Professor in the Natural Sciences

Sunny Shin, Ph.D., Associate Professor in Microbiology

Gary Wu, M.D., Ferdinand G. Weisbrod Professor in Gastroenterology

I dedicate this work to everyone who has helped me along the way.

ACKNOWLEDGMENTS

On a dark, freezing morning in 2018 I summited Mt. Kilimanjaro. During my summit trek I had over 9 hours alone on a mountain to ruminant upon experiences in my life. During that time, I specifically reflected upon the past 5 years of my life that led up to this moment and this body of work. Throughout my graduate training I have experienced the support of countless individuals. Without their love and guidance I would not be where I am today. To begin, I want to thank my thesis advisor, Dr. Rahul Kohli, for his direction and support. We may not have always seen eye-to-eye, but there was never a day that I doubted he cared about me both as a budding scientist and as an individual. For that, I am grateful. Dr. Kohli, taught me to think critically and carefully, but more importantly, he has taught me to be a scientist. I want to thank all members of the Kohli lab past and present. Specifically, I want to thank Charlie Mo for always partaking in spirited discussions and providing scientific advice. I want to thank Monica Liu for taking the time to help me grow as a scientist both at the bench and on paper. I want to thank Jeffrey Kubiak for listening and providing insightful comments. I want to thank Emily Schutsky for creating a collaborative, friendly laboratory environment and generally, always being there as a friend and colleague. I want to thank Trevor Selwood for taking the time to answer my constant biochemistry questions. And last, but not least, I want to thank Jamie DeNizio and Zach Hostetler who have been there from the beginning. As the three of us grew as scientists, we shared all our successes and failures.

I also want to thank the members of the Goulian Lab for their guidance and their willingness to always help. Specifically, I want to thank Dr. Mark Goulian for his mentorship. I also want to thank Manuela Roggiani for her patience, her expertise, and her guidance. She was instrumental in making this thesis a reality. Importantly, I want to extend my further thanks to the rest of my thesis committee, Dr. Jay Zhu, Dr. Sunny Shin, and Dr. Gary Wu. Thank you all for the stimulating discussions, the intellectual contributions, and the support both personally and professionally. I will always appreciate it.

Without a doubt, I would not be here without the love and support of family and friends. I want to deeply thank my family for always supporting and believing in me. Not a day has passed where I did not believe 100% that you all were in my corner. I want to thank my friends both in Philly and throughout the US. Thank you all for the adventures and experiences. Thank you for always supporting me, listening to me, and being there to ground me and ultimately to remind me what is truly important in life. Specifically, I want to thank Lorin Weaver for providing a listening ear, day or night, and knowing exactly what to say. I want to thank Robyn Allen and Elinor Willis my fellow VMD/PhD compatriots for their encouragement and friendship as we navigated this journey. Lastly, I want to thank Margaret O'Connor for being there when I needed it most.

Everyone in my life has contributed in some way to helping me along this journey. Climbing "Kili" I realized it truly takes a village. I am eternally grateful that my village is full of diverse, loving, and caring individuals.

ABSTRACT

NON-CANONICAL FUNCTIONS OF THE BACTERIAL SOS RESPONSE

Amanda N. Samuels
Rahul M. Kohli

DNA damage is a pervasive environmental threat, as such, most bacteria encode a network of genes called the SOS response that is poised to combat genotoxic stress. In the absence of DNA damage, the SOS response is repressed by LexA, a repressor-protease. In the presence of DNA damage, LexA undergoes a self-cleavage reaction relieving repression of SOS-controlled effector genes that promote bacterial survival. However, depending on the bacterial species, the SOS response has an expanded role beyond DNA repair, regulating genes involved in mutagenesis, virulence, persistence, and inter-species competition. Despite a plethora of research describing the significant consequences of the SOS response, it remains unknown what physiologic environments induce and require the SOS response for bacterial survival. In Chapter 2, we utilize a commensal *E. coli* strain, MP1, and established that the SOS response is critical for sustained colonization of the murine gut. Significantly, in evaluating the origin of the genotoxic stress, we found that the SOS response was nonessential for successful colonization in the absence of the endogenous gut microbiome, suggesting that competing microbes might be the source of genotoxic stress. MP1 has an antimicrobial colicin under control of the SOS response, and colicins are hypothesized to function in inter-species competition. In Chapter 3, we therefore investigated the role of colicin in promoting successful colonization of MP1. We found that in the healthy murine gut, sustained colonization does not require colicin production, thus suggesting that MP1 existing in its natural niche does not face colicin-required microbial competition. This finding calls into question the importance of colicins in an unperturbed environment.

Finally, with increased recognition of variance in SOS effectors across bacterial species, we posited that there may be a corresponding diversity in the regulatory LexA self-cleavage reaction. In Chapter 4, we systematically characterized LexA from phylogenetically-diverse bacterial species, uncovering a wide range of self-cleavage rates across bacterial species. Overall, this thesis describes non-canonical aspects of the SOS response by first exploring physiologic environments that require SOS activation, then investigating DNA repair-independent consequence of SOS induction, and finally probing the LexA self-cleavage reaction from multiple bacterial species.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

Chapter 1: Introduction

1.1 A Bacterial Survival Guide

Bacteria are single cell organisms that lack some of the complex cell regulation of higher order organisms. That said, bacteria are arguably the most successful life-form on this planet and possibly the universe. Bacteria can colonize and survive in diverse environments ranging from the soil, to the GI tract of all animals, to even hydrothermal vents. In these diverse environment's, bacteria exist in complex dynamic microbial communities that range from hundreds of bacterial cells to billions of cells depending on the given location. In these communities, bacteria face challenges from both competing microbes and from the surrounding environment. Within complex microbial communities, there is constant competition between microbes for access to space and resources. The surrounding environment poses a formidable challenge to bacteria as well. For example, bacteria living in the external environment face challenges that include UV-light, water-limitation, and temperature changes. Additionally, these natural fluctuations in the environment require rapid adjustment for bacteria to survive. As if it couldn't get more difficult, bacteria existing within or on a vertebrate or invertebrate host face challenges from the environment, other microbes, *and* from the host. In this setting, challenges from the surrounding environment can include oxygen limitation and pH changes. Challenges from the other microbes can involve direct cell-to-cell killing and secretion of bacterial-produced antimicrobials (Raffatellu, 2018). Challenges from the host can be diverse and intense including, peristalsis, oxidative and nitrosative stress, and nutrient restriction (Foster, 1999; Gibson, 2014; Begley, 2015). Overall, it is tough to be a bacterium in an ever-changing, overcrowded world. In general, the survival of bacteria largely depends upon their ability to rapidly detect, react, and adapt to challenges in the natural world.

To survive in diverse and dynamic environments, bacteria have evolved networks of genes whose protein products elegantly interact with each other to sense the surrounding environment and to coordinate a response that promotes bacterial survival and proliferation. Some of these networks come in the form of stress response pathways which typically consist of a sensor protein, a regulator protein, and the downstream effector proteins. The sensor protein detects the stress, and the regulator protein induces the downstream effector proteins. Stress response pathways largely come in two flavors, global stress response pathways and specific stress response pathways. Global stress response pathways, such as RpoS, activate large changes in metabolism and gene expression that provide protection from a variety of stresses all at once (Battesti, 2011). Specific stress response pathways have evolved to respond to generally one type of stress. These consist of a regulator and a set of genes that, when activated, are aimed at promoting bacterial survival to the given stress.

Promoting bacterial survival can take many forms. It can include fixing the damage caused by the stressor. It can involve modifying the expression levels of genes which temporarily enables bacteria to adapt to the alteration in their environment and the given stress (Galhardo, 2007). It can also encompass activating genes whose expression can modulate virulence or persistence (Fang, 2017). Lastly, it can include increasing the mutation rate to expand genetic variation enabling bacteria to permanently adapt to the selective pressure (Foster, 2005). Additionally, recent research has proposed that stress response pathways may act as an environmental sensor providing cues to bacteria about their present habitat (Cornforth, 2013). Here, it has been postulated that specific stress response pathways have evolved to enable bacteria to directly sense and respond to competition from surrounding bacteria.

In this thesis, I concentrate on one particularly significant stress response pathway, the SOS response. I will focus on critical questions involving the molecular regulators of the SOS response and the dynamic relationship of the SOS response with a complex, physiologic environment, the mammalian gut.

1.2 The SOS response

The SOS response is a highly conserved network of genes that are activated in the presence of DNA damage (Figure 1). In all bacterial species, the SOS response is tightly regulated by two highly conserved proteins, LexA, the repressor of the SOS response, and RecA, the sensor of DNA damage. In the absence of DNA damage, LexA binds to specific DNA sequences, referred to as SOS-boxes, upstream or inside of RNA-polymerase binding-sites of SOS-regulated genes. Binding of LexA to SOS boxes physically occludes RNA-polymerase from transcribing the genes, thus preventing their expression (Little, 1984; Butala, 2009). In the presence of DNA lesions, single-stranded DNA (ssDNA) is formed and sensed by RecA. When ssDNA is present in the cell, RecA filaments on the ssDNA forming a complex known as activated RecA (RecA*). RecA* induces a self-cleavage reaction in LexA and cleaved LexA can no longer effectively bind to DNA, relieving repression of the SOS-regulated genes in a coordinated fashion. A fascinating component of the SOS response is that it exhibits a time-dependent transcription of genes upon activation (Courcelle, 2001). Specifically, during elevated levels of DNA damage, the transcription of SOS-controlled genes display a temporal pattern with some genes being expressed “early” and others being expressed “late” (Culyba, 2018). When ssDNA is no longer present in the cell, RecA ceases to be active. When RecA* is no longer present, the levels of uncleaved LexA rise, thus returning the SOS response to its repressed state.

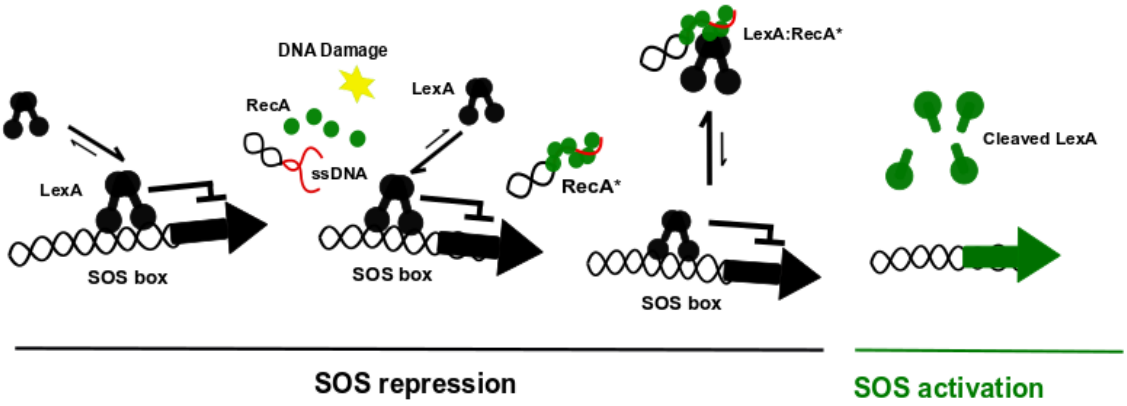


Figure 1: The SOS Response. When the SOS response is repressed, LexA (black cartoon) binds tightly to SOS boxes on DNA upstream of SOS-controlled genes, preventing their expression. In the presence of DNA damage, RecA (green circles) polymerizes on ssDNA forming the active RecA filament (RecA*). RecA* binds unbound LexA and forms the LexA:RecA* complex which promotes a LexA self-cleavage reaction that causes the dissociation of LexA from the promoters of SOS-controlled genes. Cleaved LexA can no longer bind DNA thus relieving repression of SOS-controlled genes and activating the SOS response.

Despite the conservation of the SOS response, the number and function of genes in the SOS regulon are varied across bacterial species suggesting that the SOS response may be fine-tuned to best suit the needs of a given species (Erill, 2007). For example, in *E. coli,* over 40 genes have been described to be SOS-inducible, but only 15 genes are SOS-inducible in *Pseudomonas aeruginosa* (Cirz, 2006). Other examples include *Staphylococcus aureus* and *Clostridium difficile* where 16 genes are induced by the SOS response in both bacterial species, but only 4 genes overlap (*lexA, recA, uvrB,* and *uvrA)* (Cirz, 2007; Walter, 2014). The most extreme example of SOS regulon divergence is with *Mycobacterium tuberculosis* where only 3 genes are involved in the SOS regulon, *lexA, dnaE2, and recA* (Smollett, 2012). Even though the SOS regulon varies across bacterial species, the high degree of evolutionary conservation of the SOS response highlights the importance of this response for bacterial survival amid genotoxic threats.

1.3 Activators of the SOS response

Single-stranded DNA (ssDNA) is the sole inducer of the SOS response, but the origin of ssDNA varies and can separated into three major categories: internal, environmental, and synthetic (Figure 2).

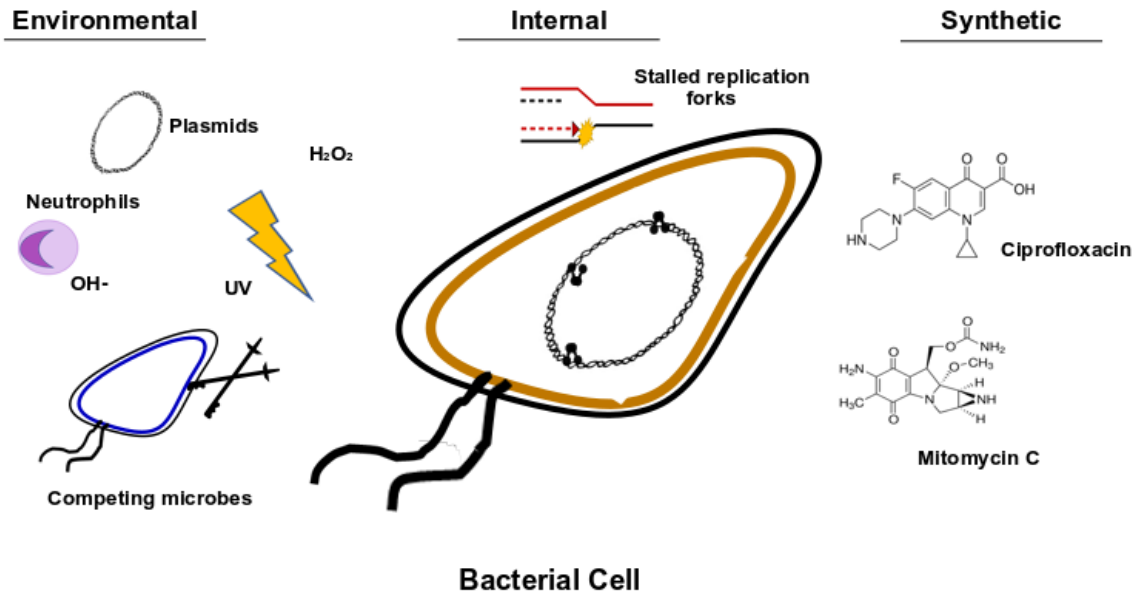


Figure 2: Representative sources of ssDNA. Bacterial cells are constantly being challenged with genotoxic stress. Environmental sources are ubiquitous and can include direct damage from OH- radicals, competing microbes, UV damage, and the uptake of DNA in the form of plasmids. Internal sources typically come from stalled replication forks. Synthetic sources are from antibiotics, such as ciprofloxacin or chemotherapeutic drugs, such as mitomycin C.

Here, internal sources of ssDNA are in reference to the formation of ssDNA in the absence of external genotoxic insults. In general, ssDNA primarily originates from stalled replication forks during replication (Pennington, 2007). In this scenario, replication fork stalling leads to double strand breaks and the formation of ssDNA on the lagging strand which provides a substrate for RecA to bind and subsequently induce the SOS response. Stalled replication forks can also come from transcription-replication collisions,

metabolic intermediates, and the formation of R-loops in nonreplicating bacteria (Mcglynn, 2012; Paul, 2013).

The formation of ssDNA due to environmental sources are numerous and the major ones are briefly discussed here. One of the best described and reliable inducers of the SOS response is UV irradiation (Oliveira, 1986). UV irradiation predominately leads to photoproduct DNA lesions in the form of pyrimidine dimers (Sinha, 2002). Reactive oxygen species (ROS) are another abundant environmental source of DNA damage. Superoxide or hydroxyl radicals can lead to DNA damage through interactions with iron and the Fenton reaction (Imlay, 1988). Additionally, hydroxyl radicals can also attack the DNA backbone thus directly inducing the SOS response (Jena, 2012). Hydroxyl radicals can also indirectly cause DNA damage through impairing proteins and lipids, which in turn can lead to increased replication fork stalling (Paez, 2011). Further, interactions with mammalian hosts can result in the formation of ROS, primarily through the action of inflammatory mediators and NO synthase-NADPH oxidases (Bernstein, 1999; Tyler, 2013; Winterbourn, 2016; Diard, 2017). DNA damage in the form of ROS can also come from direct microbial interactions (Dong, 2015). Competing microbes can also induce DNA damage through the production of antimicrobial toxins known as colicins. DNases are one class of colicins and have been demonstrated to induce the SOS response in the target cell (Vankemmelbeke, 2005; Toshima, 2007). Another commonly overlooked but critical, source of ssDNA from the environment is conjugation and transformation. In naturally competent bacteria ssDNA uptake during competency induces the SOS response and the movement of plasmids from a donor strain to a recipient strain also activates the SOS response (conjugation) (Baharoglu, 2010; Baharoglu, 2012). The consequences of DNA uptake will be discussed in the next section.

Synthetic sources of ssDNA come directly from our arsenal of tools to eliminate bacteria, antibiotics. Many antibiotics trigger the SOS response at both lethal and sub-lethal concentrations (Baharoglu, 2011). Fluoroquinolones are the best described inducers of the SOS response and act through replication arrest by blocking DNA gyrase (Pohlhaus, 2005). Additionally, some reports in the literature suggest other classes of antibiotics can indirectly cause ssDNA formation and subsequent SOS induction (Miller , 2004; Maiques, 2006; Mesak, 2008; Malachowa, 2010). Beyond antibiotics, some chemotherapeutic and anti-viral drugs can induce the SOS response. Mitomycin C is the classic chemotherapeutic drug that induces the SOS response. In fact, mitomycin C is commonly used in the laboratory to potently and reliably induce the SOS response. Other examples of SOS inducing chemotherapeutic and anti-viral drugs include 5-fluorouracil, azacytidine, and zidovudine (Oda, 1987; Bunnell, 2017).

Clearly, DNA damaging events and insults that result in the formation of ssDNA are pervasive in the environment. The diversity of these insults underscores the importance of a highly conserved and functional SOS response for bacterial survival and propagation.

1.4 Molecular activation of the SOS response

LexA self-cleavage

ssDNA provides the nidus for SOS activation, but the molecular mechanisms preceding downstream SOS gene induction are complex and have been an area of intense investigation. The SOS response is governed by the actions of two proteins, LexA and RecA. The rate of LexA self-cleavage is directly correlated to induction of the SOS response; thus, understanding the self-cleavage activity of LexA is critical to understanding activation of the SOS regulon (Mo, 2016). Mechanistic detail of LexA self-

cleavage activity has been studied primarily using two model organisms, *E. coli* and *P. aeruginosa*. LexA is a transcriptional repressor-protease that consists of two domains separated by a flexible linker. The N-terminal domain (NTD) contains specific DNA binding activity and the C-terminal domain (CTD) contains the protease activity (Luo, 2001; Zhang, 2010). The CTD contains the serine-lysine catalytic dyad and a structurally-dynamic cleavage loop that includes the target alanine-glycine bond (Luo, 2001). Cleavage at the alanine-glycine bond separates the two LexA domains, essentially inactivating LexA. Structural studies of *E. coli* LexA revealed that the cleavage loop can sample two predominate conformations; a “cleavable” form where the alanine-glycine is positioned close to the active site serine and a “non-cleavable” form where the loop is far away from the active site (Luo, 2001). Although these structural studies provide a nice “snapshot” of protein conformation, this preliminary work cannot replace thorough structure-function analysis.

In the bacterial cell, activated RecA (RecA*) mediates the self-cleavage reaction of LexA. However, *in vitro, E. coli* and *P. aeruginosa* LexA have the property of being able to self-cleave under alkaline conditions in the absence of RecA* (Little, 1984; Mo, 2014). To obtain a detailed structure-function analysis of LexA self-cleavage activity under alkaline conditions, extensive profiling of the cleavage site loop of LexA from *P. aeruginosa* was performed and both restrictive and permissive positions were identified (Mo, 2014). This study revealed important substrate preferences for LexA self-cleavage and identified single amino acid changes in the cleavage loop that either enhanced or diminished the rate of self-cleavage.

The discovery of these LexA variants raises the interesting question of whether diversity in LexA activity naturally exists among LexA proteins in other bacterial species. In a variety of reviews written about the SOS response, the question is posed as to

whether the SOS regulon has evolved to fit the specific needs of each individual bacteria (Erill, 2007; Simmons, 2008; Baharoglu, 2014). Coupled with the knowledge that variance exists in the SOS regulon across bacterial species, it is possible that LexA proteins may have evolved different rates or regulation of self-cleavage activity. In order to begin addressing this question, methodical characterization of LexA proteins from phylogenetically-diverse bacterial species should be undertaken. In Chapter 4 of this thesis, we systematically characterize the self-cleavage property of LexA from six bacterial species representing Gram-positive, Gram-negative and Mycobacterium to further elucidate whether LexA self-cleavage rate is a conserved property or represents another layer of SOS regulation.

RecA*-mediated LexA self-cleavage

Studies involving alkaline conditions have proved invaluable in terms of providing insight into the mechanism of LexA-self cleavage. However, in the bacterial cell, SOS induction does not occur in the absence of RecA* (Schuldiner, 1986; Dri, 1994). Towards that end, extensive research has been done to characterize the molecular interaction of RecA* with LexA, but the molecular mechanism governing RecA*-mediated LexA self-cleavage have yet to be fully elucidated. Parsing out the molecular interaction of RecA* with LexA is complicated by other critical functions of RecA in the cell. Outside of its interaction with LexA, RecA is responsible for mediating homologous recombination, activating mobile genetic elements including prophage, and modulating the activity of DNA pol V complex (Cox, 2007; Chen, 2008; Bell, 2016).

Multiple approaches have been used in an attempt to explain the complex interaction between LexA and RecA. To visualize the interaction between LexA and RecA*, Yu and colleagues used electron microscopy to examine the binding of LexA to RecA*. Here, cryo-EM of the RecA* filaments with a catalytically inactive LexA (K156A)

revealed that LexA may bind in the deep helical groove of RecA* (Yu, 1993). Using the crystal structure of RecA to guide residue selection, Mustard, et. al, performed site-directed mutagenesis of *E. coli* RecA in an attempt to isolate regions on RecA that specifically interfered with LexA cleavage but left other RecA functions intact (Mustard, 2000). Despite identifying mutants that reduced LexA cleavage in cells, they were unable to identify residues that only altered LexA cleavage without perturbing other RecA functions. Biochemical approaches have suggested that RecA* binding may have an allosteric effect on LexA by essentially stabilizing the cleavable conformation (Giese, 2008), yet the residues required for this interaction between LexA and RecA* have not elucidated. Based on the X-ray crystal structure of the activated RecA* nucleofilament and the highly conserved nature of RecA, Adikasavan et. al., used a computational model, Evolutionary Trace analysis, followed by site-directed mutagenesis to identify two residues on RecA that are linked to the LexA-RecA interaction (Adikesavan, 2011). From this data the authors developed a model of the LexA:RecA* complex where the CTD of LexA interacts with residues across RecA monomers and the NTD interacts with the RecA* core. In another report, Kovacic, et al. chemically cross linked the active RecA* nucleofilament to LexA and identified the cross-linked portions of the protein complex (Kovačič, 2013). Despite rigorous molecular biology, biochemical, and computational approaches no general consensus has been developed regarding the LexA:RecA* interface.

From this brief overview the complexity of the LexA:RecA* interaction is clear and developing new methodologies and approaches to studying this complex interaction is much needed.

1.5 Consequences of SOS induction

Direct DNA repair

The primary function of the SOS response is to repair DNA lesions. In most bacteria species studied, DNA repairs occur primarily through high-fidelity mechanisms followed by low-fidelity error prone repair (discussed in the next section). High fidelity repair occurs mainly through the nucleotide excision repair (NER) pathway mediated by the induction of *uvr* genes for excision of damaged nucleotides (Easton, 1983). Here, a 12-nucelotide section around the lesion is removed and the gap is filled in by DNA polymerase I and DNA ligase (Kisker, 2013). The activation of the *uvr* regulon occurs rapidly, reaching peak activity within 5-10 minutes after the genotoxic stress (Courcelle, 2001).

Genomic Plasticity

Across almost all bacterial SOS regulons investigated, SOS activation increases the expression of at least one error-prone translesion polymerase, which in *E. coli* include DinB (PolV) and the $UmuD_2$-RecA-ATP complex (PolV) (Courcelle, 2001; Cirz, 2006, 2007). The appearance of error-prone polymerases within the SOS regulon must be critical as even *Mycobacterium tuberculosis,* which has the fewest number of genes in the SOS regulon, has a translesion polymerase, DnaE2, under SOS control (Papavinasasundaram, 2001). Error prone polymerases can replicate over DNA lesions because of their wider active site and lesion recognition domain (Sale, 2013; Sharma, 2013). These polymerases aid in repair, but they also lack processivity and a proofreading domain and as such, can incorporate an incorrect nucleobase across from the lesion on the template strand (Friedberg , 2002). Consequently, incorporation of mis-paired bases throughout the genome results in a transient hypermutator state where mutation rate increases at least 1000-fold (Tompkins, 2003; Ni, 2008; Galhardo, 2009).

This transient hypermutator state, deemed SOS mutagenesis, occurs throughout the duration of the genotoxic insult.

The increase in basal mutation rate has a significant effect on bacteria evolvability providing more available genetic space in which to access adaptive mutations. A plethora of experimental and theoretical studies have demonstrated that having an increased mutation rate can result in a selective advantage in a dynamic environment (Sniegowski, 1997; Taddei, 1997; Giraud, 2001). Additionally, in the presence of DNA damaging antibiotics, SOS mutagenesis can result in increased resistance to these antibiotics (Baharoglu, 2011; Mo, 2016). Importantly, experimentally inactivating the SOS response decreases mutation rates and by extension can prevent the acquisition of antibiotic resistance (Boshoff, 2003; Cirz, 2005; Mo, 2016). There are two seminal papers that highlight this point. In one experiment Cirz et al, established a murine thigh infection model. They infected the murine thighs with either wild-type *E. coli* and or with an *E. coli* strain that had a deactivated SOS response (SOS-off). When all the mice were treated with antibiotics, both strains had an initial decline in bacterial counts, but the wild-type strain rapidly evolved resistance and the infection rebounded. The SOS-off strain, significantly, was unable to evolve resistance and the infection was cleared. In a murine infection model with *M. tuberculosis,* Boshoff et. al. inactivated DnaE2 and found that the loss of this protein reduced survival of *M. tuberculosis* and prevented the evolution of drug resistance. Taken together, SOS mutagenesis plays an invaluable role for bacterial survival and more importantly, may contribute to antibiotic resistance. However, SOS mutagenesis is not the only mechanism in which the SOS response can promote genomic plasticity.

The SOS response can also induce large genomic changes within the genome through horizontal gene transfer. In 2004, Beaber et. al. published a critical paper

describing SOS induction as leading to the activation and transfer of mobile elements in *V. cholerea* (Beaber, 2004). Here, SOS induction triggered the transfer of integrating conjugative elements (ICE) from a donor to a recipient cell. The transfer of ICE can result in acquired antibiotic resistance because these genetic elements commonly encode genes conferring resistance to chloramphenicol, trimethoprim, streptomycin, and sulfamethoxazole (Stalder, 2012). The SOS response also induces integron rearrangement and subsequent capture and expression of previously inactive genes (Guerin, 2009; Baharoglu, 2010). Briefly, integrons are genetic elements that consist of three main components: gene cassettes which are single open reading frame genes, an integrase which is the integrating unit that catalyzes the rearrangement and capture of the gene cassettes, and recombination sites that direct the specific integration of the gene cassettes. Gene cassettes largely consist of antibiotic resistance genes but can also include genes that promote bacterial survival (Gillings, 2014). Significantly, reports describing SOS activation and subsequent integron rearrangement leading to antibiotic resistance have been documented clinically (Hocquet, 2012). Terrifyingly, in this capacity, the SOS response can promote the dissemination of antibiotic resistance genes throughout a bacterial population.

Virulence and Persistence

Outside of the effects on modifying DNA, it has been increasingly recognized that the SOS response may play a role in enhancing virulence and modifying bacterial physiology. Interestingly, these functions are more specific to individual bacterial species and are briefly described here. In terms of bacterial virulence, in *Staphylococcus aureus*, SOS induction enhances expression of fibronectin binding protein, which increases bacterial tissue adherence, and can induce horizontal dissemination of virulence factors

present in pathogenicity islands (Bisognano, 2004; Úbeda, 2005). In *Listeria monocytogenes*, *E. coli*, and *P. aeruginosa*, the SOS response regulates biofilm production (Gotoh, 2010; van der Veen, 2010; Bernier, 2013). In enteropathogenic *E coli,* the SOS response regulates the locus of the enterocyte effacement (LEE), which encodes a type III secretion system responsible for secreting virulence factors into the host (Mellies, 2007). Additionally, many phage related toxins are SOS-dependent including the Shiga (Stx) toxin from *E. coli* O157:H7 (Wagner, 2001), and cholera toxin from *V. cholerae* (Kimsey, 2009). Lastly, in *Vibrio harveyi*, quorum sensing has been linked to the SOS response (Czyz, 2000).

The SOS response may also function to regulate the bacterial persistence phenotype. Persistence is a nonhereditary, reversible state where bacteria exhibit decreased metabolic activity and are essentially dormant. Persistence enables bacteria to survive an environmental insult and when that insult is removed, resume growth. Persister formation has been demonstrated to be induced by the SOS response after treatment with fluoroquinolones and depends upon SOS induction of toxins from the toxin-antitoxin family (Dorr, 2009; Dorr, 2010).

Inter-species competition

Further, SOS induction may aid in direct bacteria-bacteria interactions. In the natural environment and in the host, bacteria exist in diverse complex communities. Survival in these complex communities require bacteria to develop various defense mechanisms to acquire environmental resources and space (Hibbing, 2010). Colicins are toxins produced by *E. coli* that rapidly and specifically kill their bacterial targets (Cascales, 2007). Research into the regulation of these potent toxins has revealed an intimate connection between colicins and DNA damage (Lloubes, 1993; Mrak, 2007). Of the sequenced enteric colicin promoters, 75% have the conserved LexA binding boxes

and are regulated by the SOS response (Jerman, 2005; Gillor, 2008). Colicins are prevalent within *E. coli* species as currents estimates suggest over 50% of natural *E. coli* isolates are colicin-producing (Riley, 1992; Gordon, 1998; Gordon, 2006). Despite the prevalence of colicins, their function has not fully been elucidated. Generally, it is assumed that colicins mediate bacterial interactions, but it is unclear precisely what that means. Some models suggest that colicins enable producing bacteria to defend an environmental site against invading microbes, or to invade themselves into an established bacterial community (Streelman, 1970; Majeed, 2013). Theoretical and culture-based empirical models suggest that colicins not only promote bacterial survival, but may orchestrate local bacterial diversity through the formation of non-transitive interaction networks (Czaran, 2001; Kirkup, 2004; Walker, 2004). Models based on characterizing the types of colicin produced and the breadth of associated bacterial targets, suggest that colicins may help to mediate diversity on a larger community level (Riley, 2003). Support for this model comes from colicin-like molecules, bacteriocins. Here, it has been demonstrated that bacteriocins can alter the microbial composition of the gut when administered exogenously (Riboulet-bisson, 2012; Kommineni, 2015; Umu, 2016). The gut is a natural niche for *E. coli,* but whether *E. coli* colicins modify the microbial diversity in the gut remains unclear. A more recent model has proposed that together, colicins and the SOS response may act as an environmental signal. In this scenario, the SOS response provides a signal that ecological competition is present, colicin production occurs in some SOS-activated cells, and the remaining clonal population can escape the competitive challenge (Cornforth, 2013).

These models are attractive and colicins may function at some capacity in all the above-mentioned scenarios. However, the lack of direct testing of these models in complex physiologic environments precludes any firm understanding of the functional

role of colicins in the natural environment. To fully capture the dynamics and function of colicin production, more work needs to be done in models that mimic a natural environmental niche. In Chapter 3 we characterize the functional consequences of colicin production in a commensal *E. coli* isolate, MP1. Using this model organism, we elucidate the importance of colicin production for MP1 colonization of the murine gut where the microbial community is intact.

1.6 Environments and the SOS response

The SOS response is an effective strategy bacteria employ in order to adapt and survive. Further, the SOS response is more than a simple DNA repair pathway and may act as an environmental sensor promoting bacteria virulence and competition under times of stress. However, this raises the question: what environments necessitate a functional SOS response? Historically, understanding the mechanisms of SOS induction has revolved around a single, exogenous stressor in the laboratory, even though the pathway likely evolved to deal with a multitude of environmental stressors at the same time. Utilizing one stressor to activate the SOS response has provided a reliable model system to elucidate the molecular underpinnings of this pathway. Antibiotics or other agents that stimulate formation of reactive oxygen species have drawn more recent attention, given the intersection of the SOS response with antibiotic resistance (Imlay, 1988; Baharoglu, 2011, 2014). But these experiments do not capture what is responsible for activating the SOS response in a natural physiologic environment, nor do they provide insight into whether activation of the SOS response is required for bacteria to survive in a complex natural habitat.

Our understanding of the contribution of specific environments to SOS induction is relatively limited and largely suffers from the lack of laboratory models that mimic

natural environments. Despite this limitation, a role for the SOS response has been implicated for some pathogenic bacteria in the host. Li et. al., established that the SOS response is essential for UPEC virulence in a mouse model of human urinary tract infection (Li, 2010). In *V. cholerae,* cholera toxin (CTX) is upregulated by the SOS response (Quinones, 2005). Following this result, Quinones et. al, addressed whether the gut environment is a potent stimulus for CTX prophage induction and toxin production (Quinones, 2006). Using an infant mouse model, it was shown that after 24 hr, there was no increase in *ctxA* transcript abundance suggesting that the infant mouse intestine is not a potent stimulus for the SOS response. Further, when infant mice were co-inoculated with a wild-type and an SOS deficient mutant strain, equal numbers of both strains could be recovered at 24 hr. However, Quinones and colleagues did not extend this study beyond 24 hr and thus, it remains unclear whether CTX prophage and toxin could have been expressed and required for virulence at later time points.

The mammalian gut has been posited as an environment that might induce the SOS response. As described earlier, both host factors and competing microbes are potential sources of DNA damage (Trastoy, 2018). For example on the host side, various bile salts have been shown to increase expression of SOS induced genes *ex vivo* (Bernstein, 1999). With the pathogen *E. coli* O157:H7 the gut environment provided a DNA damaging stimulus that induced the prophage resulting in increased Stx production in a germ-free setting (Tyler, 2013). On the microbial side, Stx production increased in the presence of colicinogenic bacteria or non-pathogenic *E. coli* strains in the gut of germ-free mice (Toshima., 2007; Goswami, 2015).

While studies with pathogens suggest that the SOS response mediates some of the interplay between the mammalian gut and bacteria, the role of the SOS response in gut colonization remains unknown. Further, it is unknown whether the SOS response is

required for colonization and persistence for commensal enteric bacteria. Understanding the role of the SOS response during gut colonization is critical to not only understand the types of stressors faced by commensals as they colonize the gut but speaks to whether the gut environment more globally contributes to genomic plasticity and bacterial virulence via the SOS response. In Chapter 2 our aim was to study whether the SOS response contributes to successful bacterial colonization of the mammalian gut. In order to address this question, we needed to employ a model that captures the complex physiologic environment of the gut.

Addressing the role of the SOS response in a natural environment is complicated by the challenge of mimicking natural environments in the laboratory. Understandably laboratory models need to simplify complex environments in order to ask specific questions and reasonably interpret results, but in doing so, reduce the complexity and possibly eliminate important factors that may be critical outside of the laboratory setting. Efforts to understand *E. coli* colonization in the gut have previously relied on simplifying models. Typically, *E. coli* colonization models utilize a streptomycin-mouse model where the strain in question is engineered to be streptomycin-resistant and the mice are fed streptomycin throughout the duration of the experiment (Myhal, 1982). This model is effective for two reasons. One, it reduces colonization resistance by making available niches for *E. coli* to colonize and, two, it enables non-mouse adapted *E. coli* to the colonize the gut (Wadolkowski, 1988; Hentges, 1990). However, streptomycin alters the microbial diversity in the gut and perturbs the host immune status thus it may eliminate environmental and microbial stressors that could be relevant inducers of the SOS response (Garner, 2009; Bazett, 2016). Therefore, in order to effectively study the role of the SOS response for commensal colonization of the mammalian gut, a new model needs to be employed that captures all potentially relevant stressors.

Towards that end, our collaborators have developed a mouse model for studying *E. coli* colonization of the gastrointestinal tract that does not require continual streptomycin treatment for successful *E. coli* colonization (Lasaro, 2014). Here, a natural mouse commensal *E. coli* strain, MP1, was isolated and found to stably colonize the gut without exogenous perturbations. MP1 has a fully sequenced genome and is genetically tractable, making it an ideal strain to study the functional role of the SOS response for *E. coli* colonization. A mouse model that mimics a natural environment and an *E. coli* isolate that naturally inhabits the murine gut, together make a powerful research tool to study the SOS response and its effector proteins in the context of a complex, dynamic host environment.

1.7 Thesis objectives

The SOS response has been studied for decades. Yet, critical questions remain regarding the molecular mechanisms governing SOS regulation, the role of SOS effector genes during SOS activation in a natural environment, and what physiologic environments require an SOS response for successful bacterial survival and propagation. In this thesis, we tackle all three gaps in our knowledge. In Chapter 2, we harness the power of the *E. coli* MP1 mouse colonization model to address the role of the SOS response for bacterial colonization of the mammalian gut. We present evidence to demonstrate a critical role for the SOS response in sustained colonization of the gut and reveal that competing flora in the microbiome are a significant source of genotoxic stress. This work highlights that commensal organisms experience genomic stress in the mammalian gut and suggests that the SOS response may contribute to dynamic genomic diversification within the host microbiome. In Chapter 3, we investigate an SOS-controlled colicin. We demonstrate that MP1 produces a colicin upon SOS

induction and we characterize how this colicin promotes MP1 fitness *in vitro* and in our powerful MP1 colonization model. We show that in an unperturbed murine gut, sustained colonization does not require colicin production and suggests an unperturbed gut may not necessitate colicin-dependent inter-species competition. In Chapter 4, we systematically characterize the self-cleavage rate of the LexA protein from diverse bacterial species in both alkaline pH and in the presence of RecA*. Our results reveal a wide range of LexA self-cleavage rates across bacterial species, thus suggesting another possible layer of SOS regulation. In Chapter 5, I outline future directions that aim to build upon the conclusions of each chapter.

Chapter 2: The SOS Response Mediates Sustained Colonization of the Mammalian Gut

The contents of this chapter have been published:

Samuels, A.N., Roggiani, M., Zhu, J., Goulian, M., and Kohli, R.M. The SOS Response Mediates Sustained Colonization of the Mammalian Gut. *Infect. Immun.* **2019,** 87(2) 1-15.

2.1 Abstract

Bacteria have a remarkable ability to survive, persist, and ultimately adapt to environmental challenges. A ubiquitous environmental hazard is DNA damage and most bacteria have evolved a network of genes known as the SOS response to combat genotoxic stress. The SOS response aids in bacterial survival by regulating genes involved in DNA repair and damage tolerance. Recently, the SOS response has been shown to play an important role in bacterial pathogenesis, yet the role of the SOS response in non-pathogenic organisms and in physiological settings remains underexplored. Using a commensal *E. coli* strain, MP1, we show that the SOS response plays a vital role during colonization of the murine gut. In an unperturbed environment, the SOS-off mutant is impaired for stable colonization relative to a wild-type strain, suggesting the presence of genotoxic stress in the mouse gut. We evaluated the possible origins of genotoxic stress in the mouse gut by examining factors associated with the host versus the competing commensal organisms: In a DSS-colitis model, the SOS-off colonization defect persisted, but was not exacerbated. In contrast, in a germ-free model, the SOS-off mutant colonized with equal efficiency as the wild-type strain, suggesting that competing commensal organisms might be a significant source of genotoxic stress. This study extends our understanding of the importance of a functional

SOS response for bacterial fitness in the context of a complex physiological environment and highlights the SOS response as a possible mechanism that contributes to ongoing genomic changes in the microbiome of healthy hosts.

2.2 Introduction

Bacteria have a plethora of stress response pathways that enable them to rapidly and effectively respond to changes in their environment. One such stress response pathway, the SOS response, is activated by the presence of DNA damage and mediates bacterial survival by promoting repair of the damaged DNA (Culyba, 2015). The transcriptional repressor of the SOS response is the dual-functional repressor-protease LexA (Figure 3A). In the absence of DNA damage, LexA binds to promotors upstream of SOS-regulated genes, preventing their transcription. When DNA is damaged, RecA, the sensor of the DNA damage, is activated and stimulates LexA to undergo self-cleavage relieving its transcriptional repression of SOS-controlled genes (Courcelle 2001; Culyba, 2018). The SOS regulon varies across bacterial species, but core genes typically include those responsible for repairing or tolerating DNA damage (Erill, 2007). Importantly, DNA repair can occur in phases with early high-fidelity repair followed by lower fidelity damage tolerance pathways involving translesion DNA polymerases (Galhardo, 2009; Sale, 2013). The SOS response can also impact larger genomic changes through its regulation of conjugative elements (Beaber, 2004; Guerin, 2009). These DNA repair and diversifying functions likely contribute to the role of the SOS response in bacterial adaption to external stressors including resistance to antibiotics (Cirz, 2005; Cirz, 2006; Mo, 2016; Recacha, 2017).

Outside of its canonical function, recent research has demonstrated for a wide range of pathogens that the SOS response may play broader roles in bacterial pathogenesis and virulence. For example, SOS induction enhances expression of fibronectin binding protein in *Staphylococcus aureus* (Bisognano, 2004), contributes to biofilm production in *Pseudomonas aeruginosa* (Gotoh, 2010), and regulates Shiga and cholera toxins in *Escherichia coli* O157:H7 (Muhldorfer, 1996; Matsushiro, 1999; Zhang, 2000) and *Vibrio cholerae*, respectively (Quinones, 2005; Kimsey, 2009). Further, SOS induction may play an important role in interspecies competition that predominates in the natural environment of the host. For example, colicins are bacteriocins produced by *Enterobacteriaceae* that kill phylogenetically similar relatives and many colicins are intimately connected with the SOS response (Jerman, 2005; Mrak, 2007; Gillor, 2008). Of the sequenced enteric bacteriocin promoters over 75% of colicins are regulated by the SOS response (Gillor, 2008) and many are induced by DNA damaging antibiotics (Jerman, 2005). While much of the work has focused on pathogens, our understanding of the SOS response in commensal bacteria remains limited and there is an increasing need to fill this gap given the integral role commensals play in health and disease.

Although there is a growing recognition of more diverse roles for the SOS response, how the SOS response is induced inside a host organism remains an open area of investigation. Largely, the historical focus on this pathway has related to single and often synthetic exogenous stressor, such as UV light or antibiotics, even though the pathway has likely evolved to deal with multiple stressors naturally found in a living host environment. Studies highlighting a role for the SOS response in pathogenesis have shown that host environments can be relevant stressors for some model pathogens. In a UPEC virulence model, SOS activation increases tissue adhesin and biofilm formation (Goneau, 2015) and inactivation of the SOS response or specific SOS effectors

decreased bladder colonization in a mouse model (Justice, 2006; Li, 2010). Interestingly, while SOS activation is required for cholera toxin prophage induction *in vitro* (Quinones, 2005), in an infant mouse model of *V. cholerae* infection, induction of cholera toxin prophage was not observed. Additionally, equal numbers of a wild-type and SOS inactive strains were recovered 24 hr post infection thus leading the authors to conclude that the infant mouse gut is not a potent stimulus for the SOS response (Quinones, 2006).

The mammalian gut has been posited as an environment that might induce the SOS response. In the gut, both host factors and competing microbes are potential sources of DNA damage. For example on the host side, various bile salts increase the expression of SOS induced genes *ex vivo* (Bernstein 1999). Additionally, pathogen-associated gut inflammation in a germ-free mouse model provided a DNA damaging stimulus that induced the prophage associated with Shiga toxin (Stx) production in *E. coli* O157:H7 (Tyler, 2013). On the microbial side, Stx production is increased in the presence of colicin-producing bacteria (Toshima, 2007).

While studies with pathogens suggest that the SOS response mediates some of the interplay between the host and bacteria, the role of the SOS response in colonization remains unknown. The mediators of colonization are particularly important to decipher, as the mammalian gut is a rich environment for microbial interactions and the colonizing microbiome has been linked to both bacterial and non-bacterial diseases. To address this gap, we utilized a natural *E. coli* isolate, MP1 (Lasaro, 2014), to directly examine the role of the SOS response during sustained colonization of the murine gut in the absence of exogenous factors that could perturb host responses and microbial diversity.

We demonstrate that an SOS-off mutant is compromised relative to the wild-type strain in sustained colonization using a competitive co-colonization model. Additionally,

we addressed the relative contributions of host inflammatory responses versus the competing microbial communities in explaining the impact on colonization observed with the SOS-off mutant. Our results demonstrate the importance of the SOS response for maintenance of colonization of non-pathogenic *E. coli.* This conclusion implies that genotoxic stressors are likely continually at play in the gut and thus the SOS response could contribute to genomic and population plasticity even in a healthy microbiome.

2.3 Results

2.3.1 SOS-deficiency compromises *in vitro* fitness of MP1 in the presence of DNA damaging agents.

The role of the SOS response in complex host environments is not well established. Here, we focused on the murine gut and MP1, an *E. coli* strain that is a natural mouse colonizer and is amenable to genetic manipulation. This strain can also achieve stable colonization in the absence of continuous antibiotic treatment, a departure from the standard colonization model (Wadolkowski, 1988; Conway, 2004). This MP1 model has previously been used to establish the importance of select two-component signaling systems in sustained colonization and to demonstrate a critical role for bacterial nitrogen production in modifying the gut microbiome (Lasaro, 2014; Ni, 2017).

To decipher the impact of the SOS pathway, we engineered an MP1 derivative with its native *lexA* locus replaced with an inactive *lexA*S119A allele. LexA-S119A has a point mutation in the catalytic residue of the serine protease domain that prevents self-cleavage (Little, 1984), keeping LexA in the DNA-bound state and rendering the SOS response constitutively off (Figure 3A). To confirm this phenotype, we first examined SOS induction using a reporter plasmid which places *gfp* under the control of the SOS-

inducible *recA* promoter (Zaslaver, 2006). Using this system in the presence of ciprofloxacin as the DNA damaging agent, the wild-type showed inducible expression of GFP while SOS-off mutant shows no response (Figure 3B).

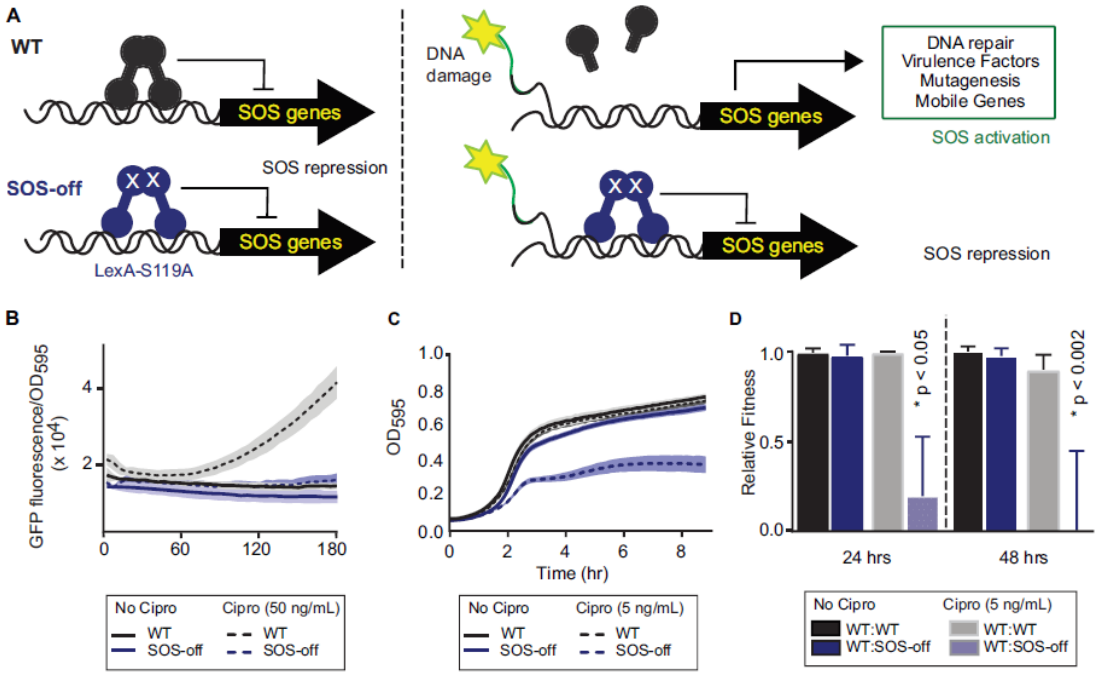


Figure 3: The SOS pathway contributes to survival in the presence of DNA damage. (A) Schematic of the SOS response. In the absence of DNA damage, LexA acts as a repressor for the SOS response. DNA damage leads to self-cleavage of LexA, activating the response in wild-type strains, but not in an SOS-off mutant with a catalytically-inactive LexA. (B) SOS reporter assay. The WT or SOS-off strains with a SOS reporter plasmid, containing GFP under the control of the *recA* promoter, were examined in the presence or absence of ciprofloxacin (Cipro). The time-dependent induction of GFP is represented as the fluorescence intensity normalized to optical density (OD_{595}) with error bands showing the standard deviations of results from three independent biological replicates for each condition. (C) Growth of cells in the absence or presence of sub-therapeutic levels of ciprofloxacin. Optical density at 595 nm was measured at 10-min intervals at 37 ºC with error bands showing the standard deviations of results from four independent biological replicates for each condition. (D) Relative fitness levels were evaluated in competition experiments between GFP or mCherry-labeled WT:WT strains or WT:SOS-off strains were evaluated in the presence or absence of ciprofloxacin at 24 hr and 48 hr. The mean fitness of each strain was calculated from two independent competition experiments. No colonies were detected in the SOS-off mutant at 48 hr with ciprofloxacin treatment, with the top of the error bar representing the limit of detection. The *P* values reported for the WT:SOS-off competition are based on a two-tailed unpaired Student's *t* test.

It has previously been established that inactivation (S119A) of LexA in *E. coli* MG1655, was associated with no measurable defect in cell growth or fitness in the absence of DNA damage (Mo, 2016). In the presence of DNA damage there was, however, a notable growth defect. We compared the wild-type and its SOS-off derivative under similar settings to determine if they demonstrated similar phenotype in MP1. In the absence of DNA damaging stress, the two strains had similar growth kinetics (Figure 3C). However, in the presence of sub-therapeutic ciprofloxacin, growth was stunted in the SOS-off mutant, but not in the wild-type (Figure 3C). To more rigorously compare the strains we performed fitness competition experiments (Lenski,1991). We introduced the SOS-off allele into a MP1 derivative marked with a tetracycline resistance cassette and mCherry under the tight control of a *tet* promoter (Lasaro, 2014). We partnered this SOS-off strain with a WT MP1 derivative containing GFP under the *tet* promoter to allow for facile discrimination of the WT and SOS-off strains in a competition experiment using these fluorescent markers. In the absence of DNA-damaging stress, no fitness defect was observed for the SOS-off mutant relative to wild-type at either 24 or 48 hr (Figure 3D). In the presence of sub-lethal DNA-damaging stress, the SOS-off mutant was significantly defective at 24 hr and completely outcompeted by the wild-type strain, with no detectable colonies, by 48 hr. Thus, as anticipated, the SOS-off mutant has comparable fitness to the wild-type strain in the absence of DNA damaging stress *in vitro* but is compromised in the presence of DNA damaging stress.

2.3.2 SOS response is important for robust growth in the mouse gut.

Establishing the reliability of the strains *in vitro* allowed us to perform competition experiments in the murine gut. Most mouse colonization studies have employed streptomycin-resistant strains of bacteria, with mice being fed streptomycin continually in their drinking water. In these experiments streptomycin aids in overcoming colonization resistance (Myhal, 1982; Hentges, 1990) and also allows for sustained expansion of strains not typically found in the mouse gut because a significant portion of the competing flora is eliminated (Hentges, 1990; Bazett, 2016). The MP1 colonization model, by contrast, employs a brief pretreatment with streptomycin for 72 hr, followed by a 24 hr washout period. Previous work has shown that normal flora rebounds within 5-6 days following streptomycin pretreatment (Antunes, 2011), allowing us to use this system to address whether sustained colonization in the gut depends upon the SOS response. Further, eliminating continual streptomycin treatment allowed us to understand the contribution of the SOS response to colonization in the absence of a confounding antibiotic stressor during the course of the experiment (Spees, 2013).

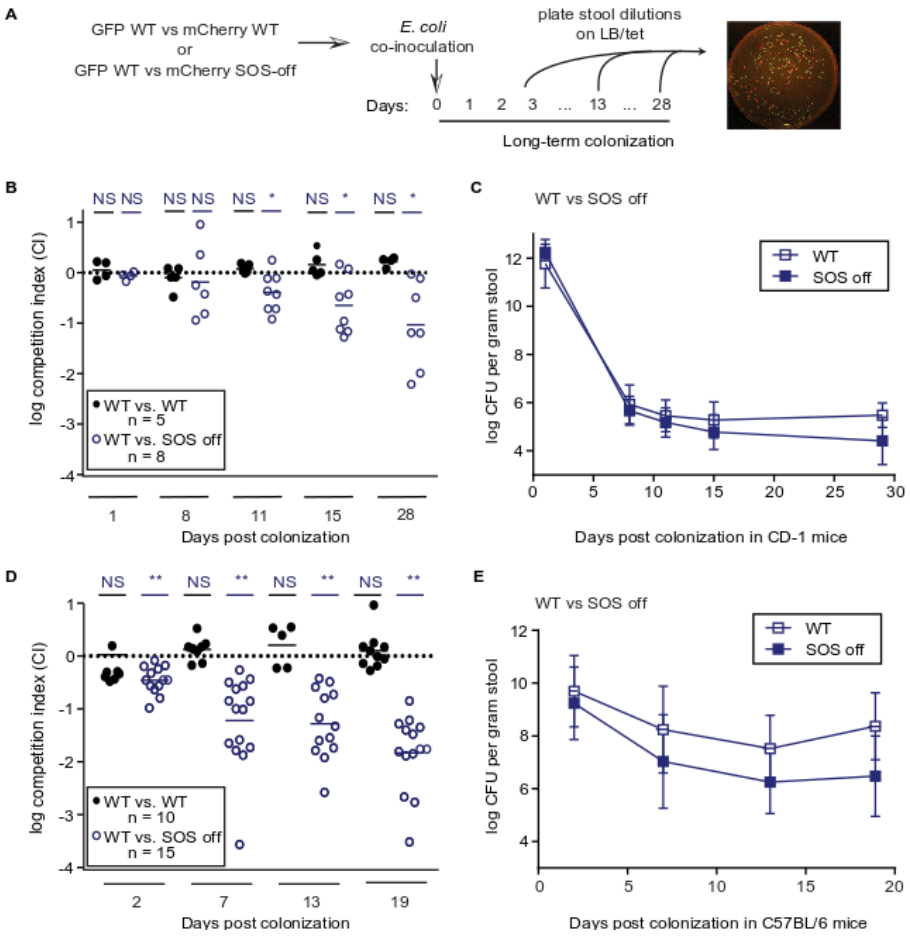


Figure 4: Colonization of the adult mouse gut. (A) Schematic of colonization protocol. Mice were inoculated with an equal mixture of *E. coli* strains and fecal samples were collected at various days post-inoculation. A representative image with GFP or mCherry marked colonies is shown. tet, tetracyline. (B to E) Groups of 6-to-8-week-old CD-1 female mice (B and C) or 6-to-8 week old C57BL/6 male mice (D and E) were co-inoculated with an equal mixture of either WT:WT strains (black filled circles) or WT:SOS-off strains (blue open circles). (B and D) Log competitive index (CI) values were calculated as the ratio of output colonies normalized to the input ratio. Each circle indicates a specific animal. Significant *p* vales are noted (NS, no significance; *, < 0.01; **, <0.001) and were calculated using one-sample *t* test. If colonies were too numerous to count on the plate that animal was excluded from the data for that day. (C and E) CFU counts of stool for the WT:SOS-off competition only. The analogous data from the WT:WT competition are shown in Fig. S2. Each square represents the mean and the standard deviation for results from all individual mice. The limit of detection was 10^2 for the CD-1 mice and 10^3 for C57BL/6 mice.

We first orally inoculated groups of CD-1 female mice with an equal mixture of a SOS-off mutant *mcherry*-marked strain and a wild-type *gfp*-marked strain. In a control cohort, we performed parallel experiments with an equal mixture of two different wild-type strains marked either with *mcherry* or with *gfp*. To understand colonization dynamics, we quantified the initial inoculum and subsequently collected feces at the start and at various days post inoculation until a set end-point. At each time point, mass-normalized feces were serially diluted and plated onto LB media containing tetracycline (Figure 4A). Tetracycline permits the selection of MP1 strains from the colonizing milieu of competing bacteria in feces and induces the expression of the fluorescent markers, permitting us to measure the total colony forming units (CFU) and the relative distribution of the GFP-marked versus mCherry-marked strains. To quantify the fitness of the strains, we calculated the competitive index (CI) by taking the ratio of input CFU counts to output CFU counts. In the control cohort, the total CFU reached levels of ~10^{12} per g stool in the immediate day following inoculation (Supplemental Figure 1). After one week, the total CFU stabilized to ~10^5, representing the establishment and maintenance of the population. These levels were maintained out to at least 4 weeks, demonstrating the strength of the MP1 model system for evaluating sustained colonization. Throughout the experimental time points examined, as expected, the GFP-marked wild-type versus mCherry-marked wild-type strains displayed equal fitness (Figure 4B). However, for the wild-type versus SOS-off mutant, significant differences emerged. The proportion of WT versus SOS-off remained non-significant up to day 8 (log CI: -0.17, ns). However, by day 11 the SOS-off mutant had a significant colonization defect (log CI: -0.4, $p > 0.01$), which increased with each subsequent time point. At day 28 the SOS-off mutant is outcompeted by the wild-type strain by over 10-fold (log CI: -1.2, $p< 0.001$), reflecting a progressive decrease in the SOS-off strain CFU, while the wild-type strain is sustained

(Figure 4C). Collectively, these experiments suggest that although the SOS-off mutant can establish colonization, it has reduced sustained colonization capacity relative to the wild-type.

Mouse strains and gender can vary in their immune responses and the composition of their microbiota. To explore the generality of our result, we next examined competition between wild-type and SOS-off mutant in a C57BL/6 male mouse model. In the control experiments with WT:WT strain competition, we observe a more modest initial drop in CFU and higher sustained colonization levels relative to the CD-1 model. The initial CFU was ~10^9 per g stool and the CFU dropped to ~10^7 but maintained those levels throughout the duration of the experiment, representing an ~10^2 higher level of colonization than observed in CD-1. Like the experiments in CD-1 mice, we captured the colonization kinetics by taking fecal samples throughout the experiment, and for each time point we determined the bacterial load and calculated the CI (Figure 4D). In C57BL/6 mice, by day 1 the SOS-off mutant was outcompeted by the wild-type (log CI: -0.45, $p<0.001$). Further, by day 7 it was outcompeted by >10-fold (log CI: -1.2, $p<0.001$). By the end-point evaluated (day 19) the SOS-off mutant was outcompeted by >65-fold (log CI: -1.9, $p< 0.001$), suggesting a greater overall impact on sustained colonization in the C57BL/6 model than in CD-1. As with the CD-1 colonization model, the CI change was a result of decreasing CFU with SOS-off mutant, rather than increasing CFU with the wild-type (Figure 4E). Although there were differences regarding total bacterial burden and the extent of competition defect, the results were similar in the two models suggesting that the reduced colonization capacity of the SOS-off mutant may not be specific to one strain or gender of mouse.

2.3.3 The SOS-off mutant can independently colonize the murine gut.

In the experimental design, the streptomycin pre-treatment is believed to open a niche to allow the MP1 strains to overcome colonization resistance. We considered whether the competition defect seen between the wild-type and SOS-off strains was a product of direct competition between the two strains in the same niches or whether the SOS-off mutant is defective for sustained colonization of the gut in isolation. To distinguish between these possibilities, two separate groups of CD-1 female mice or C57BL/6 male mice were orally inoculated with either the wild-type alone or the SOS-off mutant alone. In each system, the initial kinetics after inoculation were similar to those observed in the competition experiment, and CFU subsequently stabilized in the gut within one week. Notably, however, in both CD-1 and C57BL/6 mice, colony counts for both the wild-type and the SOS-off mutant remained stable at > 4 weeks and there was no statistical difference in colonization levels of the SOS-off mutant compared to that of the wild-type (Figure 5). Stable colonization of the SOS-off mutant alone suggests that direct competition with the wild-type occurs in the co-inoculation model and this direct competition reduces the colonization capacity of the SOS-off mutant.

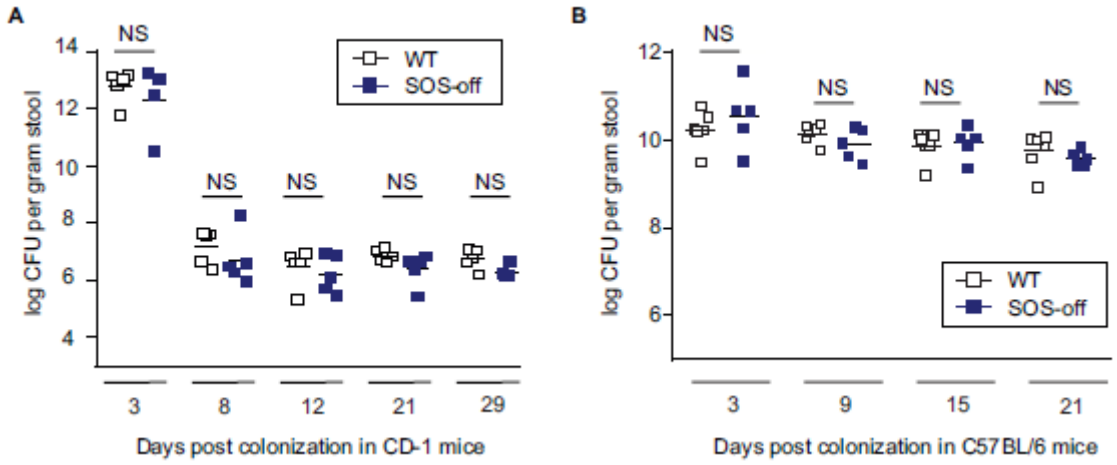


Figure 5: Solo colonization of the adult mouse gut. Groups of 6-to-8 week-old CD-1 female mice (A) or 6-to-8 week-old C57BL/6 male mice (B) were orally inoculated with either wild-type (black open squares) or the SOS-off mutant (blue filled squares). Fecal samples were collected on various days post-inoculation, and the bacterial CFU per gram of stool for each strain on a given day are shown. Each symbol represents one animal, and the limit of detection was 10^2 for CD-1 mice and 10^3 for C57BL/6 mice. NS, not statistical difference as determined by two-tailed unpaired Student *t* test.

2.3.4 Acute inflammation does not exacerbate the colonization defect.

In the *in vitro* competition experiments, in the absence of DNA damaging stress, the SOS-off mutant does not have a fitness defect relative to the wild-type strain, but in the presence of DNA damage a fitness defect manifests (Figure 4D). This implies that the SOS-off strain is subject to a source of genotoxic stress in the mouse gut that impacts sustained colonization. To examine the source of the environmental stress, we next explored two sources of genotoxic stress: host inflammation or competing commensal microbes.

We reasoned that one way to assess the impact of host inflammation would be to chemically-induce acute gut inflammation using dextran sulfate sodium (DSS). This well-established model promotes acute colitis with an increase in cytokines, chemokines, and nitric oxide, all of which could be a source of genotoxic stress for bacteria in the gut

(Beck, 2004; Yan, 2009). A major characteristic of DSS-induced inflammation is an outgrowth of Enterobacteriaceae presumably because *E. coli* can efficiently utilize nitrates and formate to outcompete other bacteria in the gut (Winter, 2013; Hughes, 2017). To examine the impact of DSS, we first pre-colonized C57BL/6 male mice equal mixtures of either the GFP-marked:mCherry-marked WT:WT or the WT:SOS-off strains. Colonization was sustained for 13 days, at which point we administered 4% DSS in the drinking water and mice could drink *ad libitum*. We collected fecal samples (Figure 6A) until the experiment was terminated due to disease activity end-point as determined by clinical criteria (Cooper, 1993). The presence of significant DSS-induced inflammation was confirmed by disease activity index, gross colon examination, and histology (Supplemental Figure 2).

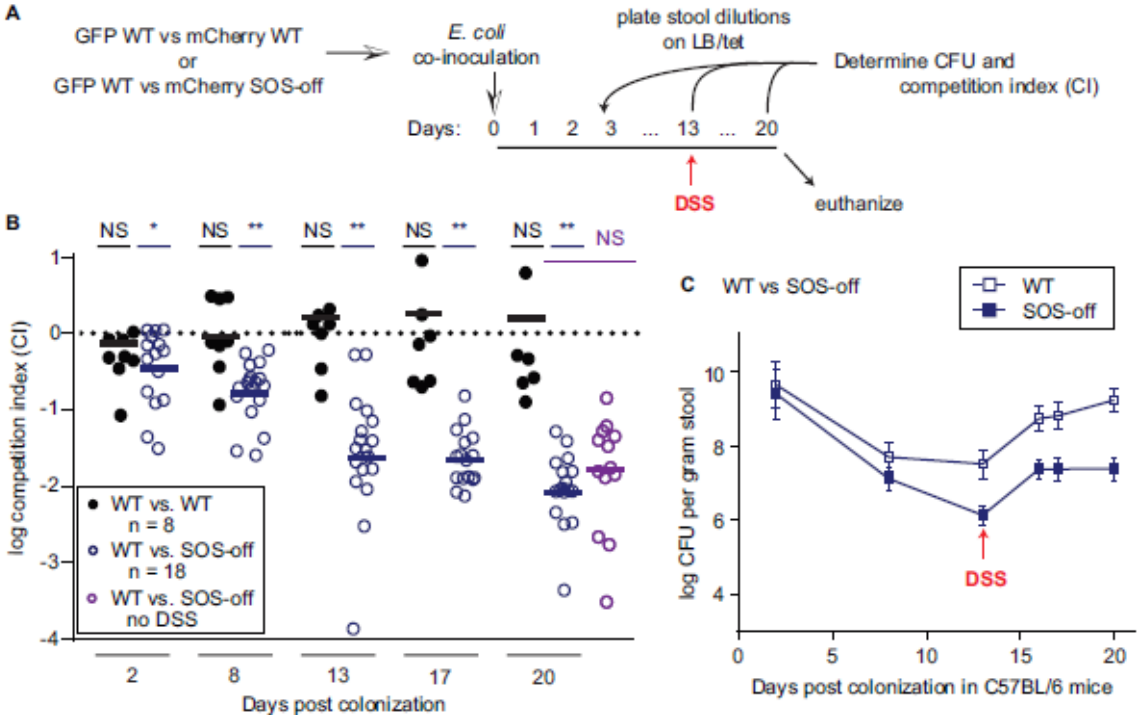


Figure 6: Effect of host inflammation on colonization. (A) Schematic of colonization protocol with DSS. Mice were inoculated with an equal mixture of either WT:WT or WT:SOS-off strains. At 13 days post inoculation 4% DSS was administered in the water. Mice were allowed to drink *ad libitum.* Fecal samples were collected at various

days until the mice were euthanized. (B) Groups of C57BL/6 male mice were inoculated with WT:WT strains (black filled circles) or WT:SOS-off strains (blue open circles). Log competition index data are shown for each competition. The purple circles represent mouse data replicated from Fig. 4 (from mice that did not receive DSS treatment) for ease of comparison. Each circle indicates a specific animal. Significant *p* values are noted (NS, no significance; *, < 0.01; **, <0.001), calculated using a one-sample *t* test. (C) Shown are the CFU per gram of stool from the WT:SOS-off competition only. The arrow indicates the start of continuous DSS administration. The analogous data from the WT:WT competition are shown in Fig. S3. Each square represents the mean and standard deviation of the results from all individual mice.

In the WT:WT control experiment, no competition was observed out to day 13 (log CI 0.2, ns) (Figure 6B). Administration of DSS resulted in a bloom of bacterial counts, with a 2-log increase in the CFU three days after DSS administration (Supplemental Figure 3), and no significant difference in the CI (log CI: 0.12, ns) (Figure 6C). In the WT:SOS-off competition, the strains tracked as expected, and by day 13 there was a ~10-fold difference in CFU (10^7 versus 10^8 log CFU/g stool), and an associated log CI -1.5, p>0.001. Importantly, after DSS administration blooms were also observed in both strains: by three days after DSS, wild-type increased CFU by ~2 logs and the SOS-off mutant also increased by ~2 logs when averaged across the observed mice. Thus, despite the induction of acute inflammation by DSS, and the associated inflammatory mediators, the SOS-off mutant was able to expand *in vivo* to a degree comparable to that of the wild-type. When the experiment was terminated due to disease burden, the wild-type outcompeted the SOS-off mutant to a greater extent than prior to DSS treatment (Figure 6B, log CI:-2.0, p> 0.001); however, the fitness defect in the inflamed gut is not statistically different from the fitness defect we observed in the healthy gut above (log CI: -2.0 and log CI: -1.9, respectively) (Figure 6B). Notably, in this experimental setup, we also aimed to confirm that the feces were an accurate reflection of colonization in the tissues. We collected cecal contents from the mice at the time of sacrifice and plated them for bacterial counts. The cecal CFU patterns observed were

consistent with those from the feces, confirming that feces are a reliable surrogate of the gut colonization (Supplemental Figure 4). Taken together, DSS-mediated inflammation thus does not amplify the defects in colonization by the SOS-off mutant to a significant extent.

2.3.5 Eliminating endogenous microbes enhances fitness of SOS-off strain.

The fact that the microbiome can dynamically shift based on metabolic conditions or disease states suggests that active competition between microbes is ongoing. As such, we hypothesized that the gut microflora could be contributing to the reduction in sustained colonization capacity of the SOS-off mutant relative to the wild-type. To test this possibility, we orally inoculated germ-free C57BL/6 mice with equal mixture of the SOS-off mutant and the wild-type and analyzed feces over ~3-week period. In this setting, colonization differs from the standard C57BL/6 model in that there is no need for streptomycin pretreatment. After inoculation, the total CFU reached levels of ~10^{12}, and only declined to levels of ~10^{10}-10^{11} over the course of the experiment (Figure 7B). In contrast to the standard model, where a time-dependent decline in the SOS-off strain was observed, in this germ-free setting, the wild-type and SOS-off strains had similar CFU counts throughout the experimental time course, and the log CI values did not differ significantly from zero, indicating there was no competition defect (Figure 7A). To confirm that the absence of a competition defect was not due to differential rates of strain shedding from the colon, we determined the CFU of wild-type and SOS-off mutant directly from cecal contents at the last time point. Once again differences were not statistically significant (Supplemental Figure 4). Thus, while the wild-type outcompetes the SOS-off mutant in a standard colonization model, having a functional SOS response is not required for sustained colonization in the germ-free model, suggesting that the

source of genotoxic stress requiring a functional SOS response is associated with an intact gut microbiome.

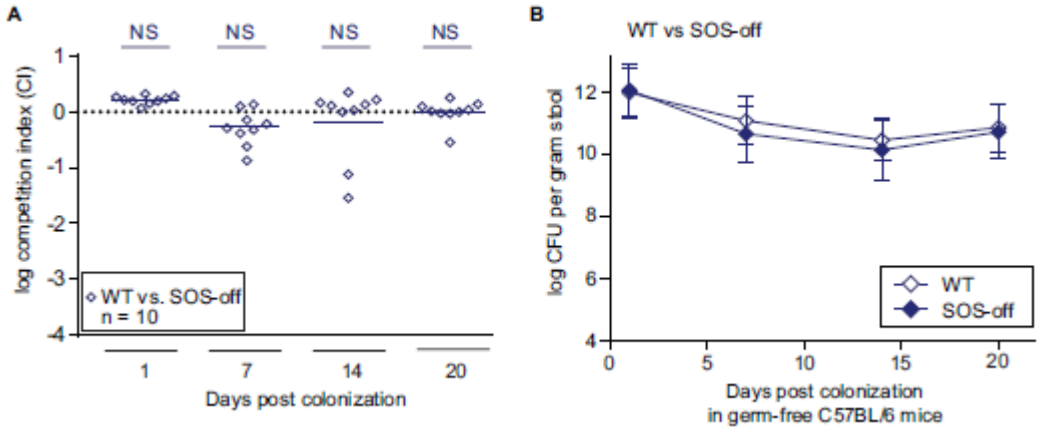


Figure 7: Colonization of the adult mouse gut in a germ-free setting. Groups of 6-to-week-old germ-free C57BL/6 mice were orally inoculated with an equal mixture of WT:SOS-off strains. Fecal samples were acquired on various days post-inoculation and at various days the (A) log competitive index and (B) CFU per g stool were determined. NS, not statistically significant as determined by one-sample *t* test.

2.4 Discussion

The SOS response is a vital stress response pathway that has long been studied in well-defined laboratory settings. These studies have been invaluable to our understanding of the molecular basis of the SOS response and its role in regulating DNA repair and genomic diversity. However, they only offer limited insight into the relationship between the SOS response and complex physiological environments. Attempts to examine the SOS response in a natural host have mainly focused on pathogens (Quinones, 2006; Li, 2010) or specific gene products of the SOS regulon (Wagner, 2001; Gamage, 2006; Justice, 2006; Bielaszewska, 2012). Collectively these studies suggest that the SOS response is an important component for successful bacterial interaction with the surrounding environment. However, knowledge of the broader role of the SOS response in a commensal strain in a host environment was lacking. Significantly, our

results demonstrate that the SOS response is important for sustained colonization of a commensal *E. coli* strain in the murine gut. In two different mouse models (CD-1 and C57BL/6) the wild-type strain outcompetes the SOS-off mutant in colonization. Interestingly, in solo colonization experiments the SOS-off mutant was recovered in statistically similar counts as the wild-type, suggesting that the competing strains occupy the same niche in this model, and that competition was important to elicit the quantitative differences between strains. This is the first report demonstrating that the SOS response is important for sustained colonization of a commensal *E. coli* strain in the murine gut.

To more thoroughly capture the dynamic process of colonization, we took a kinetic approach by monitoring bacteria burden and calculating CI continuously throughout the experiment. Taking this kinetic approach, we draw two major conclusions: First, our data imply that the bacteria are subject to low levels of DNA-damaging stress as they interact in the gut microenvironment. Data from the *in vitro* experiments suggest that the growth and fitness defect of the SOS-off mutant is only apparent in the presence of a DNA damaging agent. Notably, prior work in MG1655 demonstrates that for non-DNA damaging stressors, the SOS-off strain has comparable fitness to a wild-type strain (Mo, 2016). During solo colonization the SOS-off mutant was recovered at statistically similar CFU's as the wild-type. We posit that the fitness burden is enhanced in the presence of a wild-type strain because the wild-type strain can more effectively respond to the low-level DNA damage. Thus, in the competition setting, the SOS-off mutant can initiate colonization but cannot maintain it relative to the wild-type strain. It is possible that if the time frame of the solo colonization experiment had been extended there would have been an eventual decline in SOS-off colony counts in the absence of competition.

Second, the colonization kinetics suggest that the SOS response might be more important for maintaining colonization than for initiating colonization. In the competition experiments, although kinetics differed slightly between CD-1 and C57BL/6, both the wild-type and the SOS-off mutant were recovered throughout the experiments, but there was a subsequent time-dependent decline of the SOS-off mutant CFU. The more limited effect during the first 24 hr aligns with a prior results in *Vibrio cholerae* (Quinones, 2006), where wild-type and a SOS-off *V. cholera* were recovered in equal numbers at 24 hrs. Such limitations in distinguishing initiation versus maintenance effects have previously also been observed in the study of nitric oxide associated stress in the *V. chloerae* infant mouse model (Stern, 2012). The results of short-term colonization with *V. cholerae* and in our study, however, differ from those observed with UPEC, where initial colonization of the urinary tract was compromised in an SOS-deficient UPEC strain (Justice, 2006; Li, 2010). These distinct findings illustrate that different host environments may have different SOS response requirements. As important, these findings highlight potential differences in mechanisms that might be at play in pathogens versus commensal.

For a resident microbe, the gut is a complex environment where there is an interplay between host factors and competing microbes. To examine the effect of perturbing the host environment we used DSS to induce inflammation. Inflammation is often associated with an increase in DNA damaging stressors such as reactive nitrogen, oxygen, and hypochlorite, and DSS has been linked to inducible nitric oxide synthetase as bacteria breach the mucus layer (Johansson, 2010; Perse, 2012). However, DSS-induced inflammation had no effect on competition. We speculate that either the strains are not directly subject to this associated inflammatory response, or as a host-adapted strain they may have evolved means to adequately handle these inflammatory responses independent of the SOS response. In this regard, prior work presents some

stimulating results for further consideration in the context of our studies. In *Salmonella*, an increase in phage transfer was noted to be dependent upon inflammation-induced SOS response (Stecher, 2012; Diard, 2017). Interestingly, when mice defective in either NO synthase-NADPH oxidase or myeloperoxidase were examined, reactive oxygen species, nitrogen species, and hypochlorite production are decreased, yet phage transfer occurred at similar levels to those observed the wild-type mice. These results suggest that SOS-inducing stress associated with inflammation could be coming from alternative sources, including competing microbes, rather than the host itself.

In germ-free mice, no competitive advantage was observed for the wild-type relative to the SOS-off mutant. We envision at least two scenarios that can explain this result, which are not mutually exclusive. First, given the absence of established competing flora in the germ-free model, the *E. coli* strains expanded into niches where host sources of DNA damage are minimal. In line with this possibility, the higher levels of colonization suggest the possibility that alternative niches not present in the standard model could dominate the population counts in the germ-free model making the fitness defect undetectable. Second, it is possible that competing gut microbes are a potential source of genotoxic stress in the standard model and absent in the germ-free model. This explanation aligns with prior literature where SOS-controlled effectors, such as Shiga toxin, have increased production in the presence of other microbes (Toshima, 2007). The fact that the defect in the SOS-off mutant was only apparent in the setting of competition experiments in the intact microbiome model suggests that different factors may be at play when a narrow niche is opened by streptomycin-pretreatment in the standard model versus the wide-open sterile gut in gnotobiotic experiments. These possible differences have implications for the streptomycin-treated mouse model, where streptomycin is maintained throughout the duration of the experiment to sustain a

colonizing strain. As streptomycin eliminates a significant portion of the microbial diversity in the gut (Hentges, 1990), this model risks masking possible contributions from microbes that are eliminated by this sustained selection.

The SOS response provides many diverse functions for the bacteria and, consequently, multiple effectors could be contributing to sustained colonization of the gut. Given its canonical function in DNA damage repair and tolerance, the inability to rapidly repair the damage could contribute to the fitness defect of the SOS-off mutant. However, non-canonical functions could also be relevant in the colonization model. Interestingly, prior work in the streptomycin-treated model has suggested that colicin-producing bacteria better sustain long-term colonization of the mouse gut (Gillor, 2009). MP1 harbors a plasmid with a colicin gene that contains LexA-binding motifs in its promoter. While we did not aim to isolate the key SOS effectors that mediate sustained colonization, it is conceivable that colicins may play a role. Moreover, given the diverse and complex nature of the SOS regulon it is probable that multiple SOS controlled effector proteins are responsible for the colonization defect of the SOS-off mutant.

Our results indicate that the SOS response contributes to full-fitness of a commensal bacterium in the context of a natural gut environment. Beyond its implications in colonization, this finding has added significance with regards to the generation of genomic diversity in the gut. The SOS response is known to be a major driver of mutagenesis via its DNA damage tolerance mechanisms and is associated with the movement of larger blocks of DNA via control over phage induction and integrons (Guerin, 2009; Hocquet, 2012). Our results imply that commensal bacteria are subject to genotoxic stress that requires activation of the SOS response, even in the healthy microbiome. In the absence of external stressors such as antibiotics, these commensal organisms may still be triggering genome-diversifying activities that could promote the

acquisition of mutations or genes associated with antibiotic resistance. Thus, the relevance of stress responses and potential opportunities for targeting these responses clinically could extend beyond acute infections and to chronic settings that may be predisposed for infections to occur.

2.5 Materials and Methods

2.5.1 Congenic strain generation. The GFP- or mCherry-marked MP1 strains, also known as MP7 and MP13, were previously described and used for strain construction and competition experiments (Lasaro *et al.*, 2014). The SOS-off mutant was constructed in two steps, using the close linkage of *lexA* with *malE*. First, *malE* was deleted using P1vir transduction from the Keio collection. Strains were confirmed by PCR and their inability to grow in the presence of maltose as the only carbon source. Second, a previously generated MG1655 *lexA*S119A strain, encoding LexA with a mutation in the catalytic serine that renders it non-cleavable, was used to introduce the mutant *lexA* allele (Mo, 2016). The *lexA*S119A allele was introduced into the Δ*malE* strain by P1vir transduction from lysate derived from the MG1655 *lexA*S119A. Strains with restored *malE* gene were verified by their ability to grow on maltose as the only carbon source and integration of the lexA*S119A* allele was confirmed by PCR and sequencing. Strains are available upon request.

2.5.2 *In vitro* assays. SOS reporter assay. Induction of the SOS response was monitored by reporter plasmids containing GFP under the control of the *recA* promoter as described previously (Culyba, 2018). Briefly, bacteria were transformed with GFP-reporter plasmids and cultured in defined media containing 1 x M9 salts (Sigma M6030), 0.4% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.05% casamino acids and 30 µg/mL of

kanamycin to maintain the plasmid. Overnight cultures were diluted 1000-fold into fresh media and incubated with shaking at 37 ºC and grown until absorbance at 595 nm (OD_{595}) ~0.3. At this point 100 µL aliquots were dispended into a 96-well, round bottom, transparent plate. DNA damage was induced with 50 ng/mL of ciprofloxacin dissolved in PBS and an equivalent amount of PBS was added to control wells. The plates were incubated at 37 ºC and GFP fluorescence intensity (RFU) and OD_{595} were acquired every 5 minutes for 180 min on a Tecan Infinite F200 Pro multi-functional plate reader, agitating before every data acquisition cycle.

Growth rates were measured as described previously (Mo, 2016). Briefly, overnight cultures were diluted 1000-fold into fresh LB in the presence or absence of sublethal concentrations of ciprofloxacin (5 ng/mL) and distributed into 96-well, round bottom, transparent plates. Cultures were incubated at 37 ºC with cycled agitation and OD_{595} measurements were taken continuously.

Competition assay was adapted from established protocols (Mo, 2016). Briefly, overnight cultures of the *gfp* or *mcherry* tagged strains were standardized by optical density. The strains were mixed at a 1:1 ratio and diluted 10^6-fold in 3 mL of LB in either the absence or the presence of ciprofloxacin (5 ng/mL). The co-culture was incubated overnight at 37 ºC with aeration for 24 hr. The next day, the overnight culture was diluted 10^6-fold and re-inoculated into fresh LB and grown for an additional 24 hr. To determine the CFU of each strain, cultures samples were taken at time zero, after 24 hr, and after the 48 hr growth period, plated onto LB agar + 15 µg/mL of tetracycline and incubated overnight at 37 ºC. Plates were imaged using a previously described system that permits detection of GFP and mCherry (Siryaporn, 2008). The relative fitness was then calculated by comparison of the starting population and the population at each time point, according to the formula of Lenski and coworkers (Lenski, 1991).

2.5.3 Competition or solo colonization experiments. All animal studies were carried out in accordance with the Institutional Animal Care and Use Committee of the University of Pennsylvania. Animal protocols followed the guidelines established within the "Guide for the Care and Use of Laboratory Animals" (8^{th} ed.) published by the National Research Council of the National Academies.

Experiments were performed with 6-8-week-old pathogen-free CD-1 female mice purchased from Jackson Laboratories or pathogen-free C57BL/6 male mice purchased from Charles River Laboratories. Germ-free C57BL/6 mice were maintained in plastic isolator units and fed autoclaved chow and water. Each cage contained 4-5 mice. In the standard protocol, to overcome colonization resistance the mice were provided 5 g/L streptomycin and glucose in their drinking water for 72 hr. Fresh water, without antibiotic and glucose, was then given to the mice for 24 hr prior to oral inoculation with *E. coli* strains, and the mice were maintained on antibiotic- and glucose-free water for the remainder of the experiment. For germ-free experiments, no streptomycin pretreatment was performed. For the inoculum, bacterial cells were prepared by picking a single colony from an LB agar plate and grown overnight with aeration at 37 ºC in LB. The following day, OD_{595} was measured using a 1:10 dilution of the overnight culture and the concentration of cells was calculated. Cells were spun down at 3800 x *g* at 4 ºC and resuspended in cold phosphate-buffered saline (PBS). Cells were washed twice with PBS and after the final wash cells were resuspended in a volume of PBS that equaled ~10^{10}-10^{11} cells/mL. To start the competition experiment, the cell suspensions were mixed 1:1 and mice were orally inoculated by gavage with 100 µL of the mixture. Solo colonization experiments were performed by inoculating with 100 µL of the bacterial suspension. A portion of the inoculum was serially diluted and plated on LB agar with 15

µg/mL of tetracycline to determine the input CFU. Throughout the experiments in standard conditions, mice were raised on standard laboratory rodent diet (LabDiet 5001). For experiments with the colitis model, at day 13 mice were given 4% dextran sulfate sodium (DSS: Affymetrix molecular weight 40-50 kDa) in water and could drink *ad libitum*. Disease activity score was monitored daily as described previously (Cooper, 1993). Disease activity score determined when the mice were euthanized. For histopathology on the DSS-treated and not treated mice, tissues were fixed in formalin and then processed by the University of Pennsylvania Comparative Pathology Core. Samples were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and reviewed by a veterinary pathologist.

2.5.4 Determination of *E. coli* CFU. At each time point of interest, 3-4 stool pellets were obtained from each mouse. The fresh feces were weighed and resuspended as a slurry in PBS to the final concentration of roughly 0.5 g of feces per 1 mL PBS. The samples were serially diluted and plated on LB with 15 µg/mL tetracycline. Fluorescence images of plates were obtained as described above. The competitive index (CI) was determined as [(mCherry fluorescent CFU/(GFP fluorescent CFU)]/[(input mCherry CFU)/(input GFP CFU)] where the input CFU was determined from the inoculum.

2.6.1 Acknowledgements

The authors thank Charlie Mo and the members of the laboratories of R.M.K and M.G. for helpful discussions and critical reading of the manuscript. We thank Josie Ni and staff members of the University of Pennsylvania DLAR for help with animal studies. We acknowledge the technical support provided by Dmitri Kobuley and the University of Pennsylvania Germ-Free Mouse Core. This work was supported by NIH grants R01-GM127593 (to R.M.K) and R21AI125814 (to M.G and J.Z), along with the Burroughs

Wellcome Foundation Investigators in the Pathogenesis of Infectious Disease Award (to R.M.K). The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication. The authors declare no financial conflict of interest.

Chapter 3: The Role of Colicins During Commensal *E. coli* Colonization of the Mammalian Gut

The contents of this chapter are currently being prepared as a manuscript for publication.

3.1 Abstract

Colicins are specific and potent toxins produced by *Enterobacteriaceae* that result in the rapid elimination of sensitive cells. Colicin toxins are ubiquitously found throughout microbial species, suggesting their importance for bacterial survival in complex microbial environments. Colicin biology has been predominately studied using theoretical and synthetic laboratory models and it is unclear how colicin production contributes to survival and fitness of a colicin producing commensal strain in a physiologic environment. To address this gap, we took advantage of a natural *E. coli* commensal strain, MP1, that produces a colicin toxin. We found that MP1 colicin was regulated by the SOS response and was active against a phylogenetically diverse *E. coli,* Nissile 1917. MP1 is a natural colonizer of the murine gut, and thus can stably colonize the gut without perturbing the indigenous gut microbiome. Using this model, we directly interrogated the importance of colicin for MP1 survival in the gut. We showed that in a healthy gut with an intact microbiome, sustained colonization does not require colicin production. Our results suggest that colicin-mediated responses may not be critical as commensals interact with a gut microenvironment. This report extends our

understanding of the function of colicins during commensal colonization and calls into question the importance of colicins for a commensal living in its physiologic niche.

3.2 Introduction

In most natural environments' bacteria exist in diverse, highly populated, and complex communities. For example, in a gram of soil there are roughly 10^{10} bacterial cells with a species diversity of at least 4 x 10^3 (Torsvik, 1990). The numbers and diversity are even more striking in the human colon where the current estimate of bacterial density is approximately 3.8 x 10^{13} and the diversity is greater than 1000 species (Lozupone, 2012; Sender, 2016). In order to survive in these complex microbial microenvironments, bacteria are engaged in a fierce competition for space and resources (Hibbing, 2010; Stubbendieck, 2016). To respond to competitive challenges, bacteria have evolved numerous and varied mechanisms to survive. Production of antimicrobial molecules is one mechanism used throughout the microbial kingdom to inhibit the growth and limit the survival of competitors (Czaran, 2001; Wloch-salamon, 2008). These antimicrobial molecules can directly target phylogenetically-different species, related strains of the same species, or even genetically identical bacteria (Claverys, 2007; Be'er, 2009). One specific class of antimicrobials are bacteriocins, which are one of the most abundant and diverse classes of molecules found throughout the bacterial kingdom. It is estimated that 99% of all bacteria synthesize at least one bacteriocin and in some bacterial species multiple bacteriocins are produced (Klaenhammer, 1988; Riley, 1992). Bacteriocins are toxins that have a very specific host range and are generally lethal to strains competing for similar resources (Riley , 2002).

Escherichia coli produce a specific class of bacteriocins called colicins (Cascales, 2007). Colicin genes are found within genomic clusters on colicinogenic plasmids. These clusters contain the colicin gene, an immunity gene which confers resistance to the cell against its own colicin, and a lysis gene which aids in colicin release (Cascales, 2007). Colicins are incredibly prevalent in a microbial population. Recent estimates have suggested that in a given human fecal sample at least half of the *E. coli* strains will produce a type of colicin and some of those strains will produce more than one colicin (Gordon, 2006). In a survey of feral house mice, 400 isolates of *E. coli* were identified and of these strains, over 50% harbored colicinogenic plasmids (Gordon, 1998). The high frequency with which colicins are encountered in nature, and specifically in the gut, suggest that colicins are vital to microbial communities, but the precise functions remain unclear.

Colicins have been proposed to promote the survival of the colicin-producing strain and, simultaneously, modulate the broader microbial community. The specificity of colicin targets suggest they may aid in promoting *E. coli* niche survival in the intestinal microbiota (Hibbing, 2010), enabling it to survive in a highly competitive environment (Riley, 1999). *In vitro* experiments have demonstrated that colicin expression is tightly regulated by nutrient limitation or the SOS response suggesting that colicin production is critical during times of stress when competition for resources is even more limiting (Gillor, 2008; Ghazaryan, 2014) Laboratory-based studies have suggested that colicin production may limit the invasion of competing microbes into an occupied niche (Kerr, 2002). Further, colicin production in the setting of an inflammatory environment provides a competitive advantage to the invading colicin-producing strain, enabling it to displace a colicin-sensitive strain (Nedialkova, 2014). However, these studies do not address

whether colicin production contributes to *E. coli* survival and persistence in a stable physiologic niche.

The mammalian gut is characterized by a high density and diversity of microbes all of which co-exist in a natural equilibrium thus making it an ideal ecosystem to investigate the functional consequences of colicin production for sustained colonization. In that regard, few empirical studies have been published to elucidate the role of colicin production for successful *E. coli* colonization in the unperturbed mammalian gut. In a study evaluating colonization and *Enterococcus faecalis,* a pheromone-responsive conjugative plasmid encoding bacteriocin 21 was demonstrated to be important in colonization, outcompeting *E. facecalis* strains that lacked the bacteriocin (Kommineni, 2015). In another study, streptomycin-treated mice were inoculated with either a colicin-sensitive *E. coli* strain, a colicin-producing *E. coli* strain, or a colicin-resistant *E. coli* strain. The mice were co-housed, and strain dynamics were monitored. In this model, the colicin-producing strain generally out-competed the other strains (Kirkup, 2004). In another streptomycin-treated mouse model, colicin-producing *E. coli* strains had increased persistence in the mammalian gut compared to non-colicin-producing *E. coli* strains (Gillor, 2009). Further, in that study, colicin-producing strains also maintained significantly higher densities than the non-colicin producing strains.

Taken together these studies imply a significant role for colicins for bacterial colonization of the mammalian gut. However, for both *E. coli* colonization models, the experiments deviate from a natural setting in two fundamental ways. First, the mice were treated with streptomycin throughout the duration of the experiment. For most colonization models, streptomycin pretreatment is necessary to overcome colonization resistance, but it reduces microbial diversity and alters the mouse immune status (Hentges, 1990; Bazett, 2016). Second, *E. coli* strains used in this study are not natural

colicin-producing isolates. Engineering naturally-sensitive *E. coli* strains to produce colicins could have unknown consequences to bacteria physiology. Therefore, it is still not clear whether colicin production is a requirement for a commensal living in its natural niche in an unperturbed environment.

In this study we aimed to characterize the functional consequences of colicin production in a natural enteric commensal *E. coli*, MP1. Commensal bacteria are instrumental in regulating gut homeostasis; however, the molecular effectors commensals utilize in order to interact with the surrounding microbial community and host remain largely unclear. Deciphering these effectors in the context of a healthy mammalian gut is critical given the critical role of the microbiome in preventing both bacterial and nonbacterial associated diseases. MP1 is a natural mouse isolate and can sustain colonization in the gut in the absence of exogenous antibiotic treatment (Lasaro, 2014). MP1 has a naturally occurring plasmid-encoded colicin genomic cluster, thus making it an ideal model to examine the role of colicin in promoting sustained colonization in an unperturbed environmental niche.

We found that MP1 colicin is regulated by the SOS response, a DNA-damage inducible stress response pathway, thus suggesting that MP1 colicin is expressed during times of genomic stress. MP1 has a narrow spectrum of activity, similar to other characterized colicins (Gillor, 2008), but this activity is against a phylogenetically distant *Enterobacteriaceae.* Importantly, we identified a natural human *E. coli* isolate, Nissle 1917 as being sensitive to MP1 colicin. In head-to-head *in vitro* competition experiments, wild-type MP1 outcompeted Nissle 1917, and this fitness advantage was dependent upon colicin production. After establishing the importance of colicin production *in vitro,* we moved to a natural *in vivo* model. We first asked whether colicin production of the wild-type MP1 strain provided a colonization advantage in competition with a colicin-

deficient, yet resistant, MP1 strain. In this model, colicin production did not provide a fitness advantage, and both strains co-existed throughout the duration of the experiment. Further, the colicin deficient strain was capable of solo colonizing the murine gut, suggesting that in the gut of a healthy mouse, colicin production is not required for sustained colonization. Our work suggests that a commensal *E. coli* living in its ecological niche may not encounter microbial competition that necessitate colicin production. Our work lays the groundwork for further exploration into the environments that require colicin production for bacterial survival.

3.3 Results

3.3.1 The SOS response regulates colicin production in a commensal *E. coli* strain, MP1.

Previous work by our group identified the murine commensal *E. coli*, MP1, as harboring a 8.5-kb plasmid that carries a colicin gene with substantial homology to the previously-characterized colicin genes, colicin Y and colicin U (Table 1) thus suggesting MP1 colicin is a pore-forming colicin (Riley, 2000; Lasaro, 2014).

	COLICIN Y	***COLICIN U***
COLICIN PROTEIN	99.5%	99%

Table 1: Amino-acid sequence identity of MP1 colicin with closely related colicins, U and Y

Colicin production is tightly regulated and often only produced during times of stress (Ghazaryan, 2014). Therefore, we wanted to first investigate MP1 colicin regulation in order to understand what environmental stressors might precipitate colicin production in MP1. In *E. coli*, the expression of many colicins is triggered by DNA damage due to

regulation by the SOS response (Jerman, 2005; Cascales, 2007; Gillor, 2008). In the absence of DNA damage, transcription of SOS-controlled genes is repressed by LexA, the regulator of the SOS response. In the presence of DNA damage, LexA repression is relieved and transcription can occur. Genetic analysis of the promoter region of the MP1 colicin gene showed two overlapping canonical SOS-boxes where LexA could bind (Figure 8A). This suggests the possibility that MP1 colicin is regulated by the SOS response and is only produced in the presence of genotoxic stress. Additionally, MP1 colicin promoter region contains an IscR binding site upstream of the overlapping SOS-boxes, suggesting another possible layer of colicin regulation.

We wanted to confirm whether DNA damage influenced colicin expression in MP1. To do this, we engineered a reporter plasmid which places the green fluorescent protein gene (*gfp*) under the control of the promoter region of the MP1 colicin gene. As a control, we placed *gfp* under the regulation of another SOS-inducible promoter (that of the *dinB* gene) (Zaslaver, 2006). In the absence of UV light (50 J/m^2) as the DNA-damaging agent, there was no expression of GFP whereas in the presence of UV light there was inducible expression of GFP (Figure 8B). The GFP expression pattern of the *colicin* promoter mirrors the expression pattern of the *dinB* promoter, suggesting there may be a delay in *colicin* induction from the initial activation of the SOS response. SOS-controlled genes display time-dependent kinetics with some genes being induced early in the SOS response, such as *recA*, and others being induced late in the response, such as *dinB* (Courcelle, 2001; Culyba, 2018). The timing of GFP expression suggests that MP1 colicin expression is expressed later in the SOS response which is consistent with previous analysis of SOS-controlled colicins (Herschman, 1967) To verify the timing kinetics of the colicin promoter, we repeated the experiment in the presence of the SOS-inducible promoter, *recA,* which is expressed early in the activation of the SOS response

(Supplemental Figure 5). GFP induction occurred by 25 min for the *recA* promoter and 35 min for the *colicin* promoter reinforcing the conclusion that colicin production occurs later in the SOS response. For some colicins the delayed expression is aided by IscR (Iron-sulphur cluster regulator) which stabilizes LexA dimers at the promoter, delaying the expression of colicin until there is also a depletion of nutrients (Butala *et al.*, 2012). Since MP1 colicin has an IscR binding site upstream of the SOS-boxes this suggests that this mechanism may be relevant in MP1.

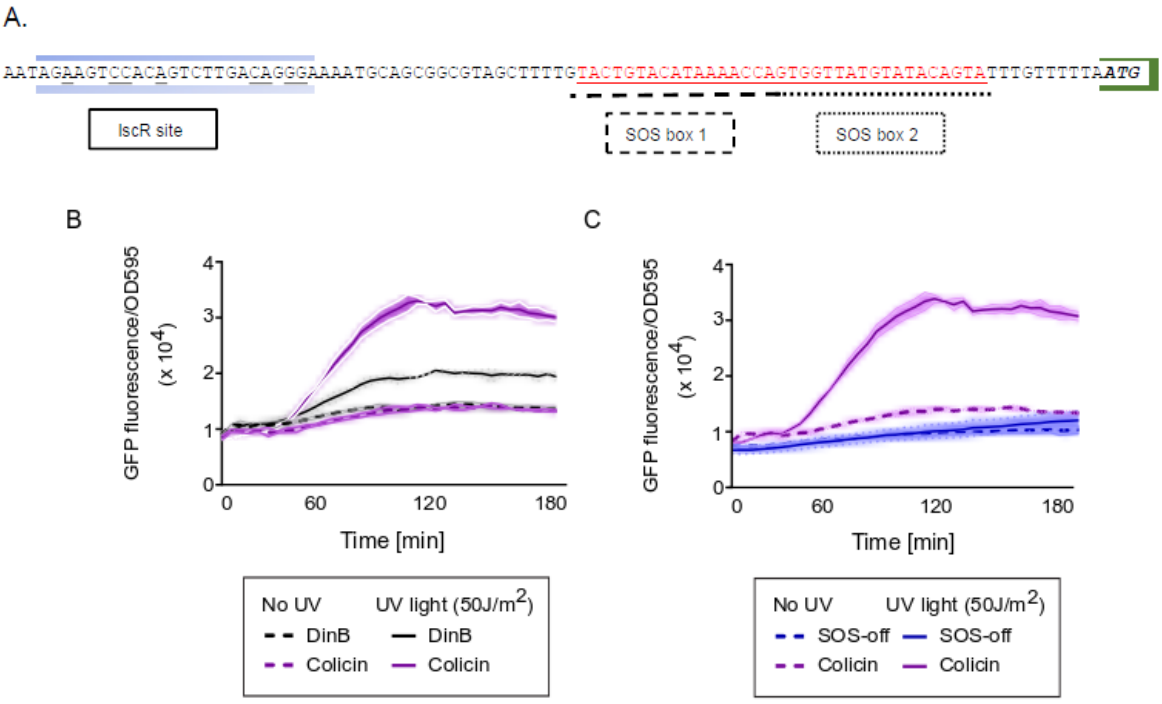


Figure 8: SOS regulation of MP1 colicin. MP1 production of colicin is SOS regulated (A) sequence analysis revealed two overlapping SOS boxes upstream of the ribosome binding sequence and start codon. (B) DNA damage reporter assay. WT strains with a SOS reporter plasmid, containing GFP under the control of *colicin* promoter or *dinB* promoter were examined in the presence or absence of UV light. (C) SOS-dependent reporter assay. The WT or SOS-off strain with the *colicin* promoter SOS reporter plasmid were both examined in the presence or absence of UV light. For both GFP experiments, the time-dependent induction of GFP is represented as fluorescence intensity normalized to optical density at 595 nm. The error bands show the standard deviations of results from three independent biological replicates for each condition.

To confirm that the expression of *gfp* from the colicin promoter was due to LexA cleavage and SOS induction, we utilized a previously-constructed MP1 strain that cannot activate the SOS response: SOS-off (Samuels, 2019). This strain encodes a catalytically-inactive LexA protein via a single point mutation that prevents the LexA cleavage reaction, keeping LexA in the DNA-bound repressor state and effectively repressing the SOS regulon (Little, 1984; Mo, 2016). This mutant strain was transformed with the *gfp*-colicin promoter construct and GFP fluorescence was monitored in the presence and absence of UV light (50 J/m^2) (Figure 8C). As expected, the SOS-off strain showed no induction of GFP in either condition, thus indicating that colicin production is dependent upon DNA damage and the SOS response. In the absence of UV light, there was slightly elevated GFP expression in the wild-type strain relative to the SOS-off strain. This is explained by the basal level of activity commonly seen with SOS-regulated genes due to spontaneous induction of the SOS response as a result of double-strand breaks (Pennington, 2007). Further, this result confirms the tight repression of LexA-controlled genes in the presence of the catalytically-inactive LexA mutant.

3.3.2 MP1 colicin is active and depends upon a functional SOS response.

We next wanted to demonstrate that MP1 expressed an active colicin protein. To do this, we induced colicin production in wild-type MP1 by UV light and spot plated serial dilutions of cell lysate on soft agar plates containing laboratory strain MG1655. The zones of inhibition were determined by scoring for either clear zones for complete inhibition or turbid zones for partial growth inhibition. In the absence of UV light exposure, basal levels of colicin production were observed as there was turbid zone of growth only for the undiluted colicin sample (Figure 9A). This result was expected because of the basal levels of colicin produced in a population as discussed previously

(Pennington, 2007). After UV light exposure, however, clear zones of inhibition were seen up to a dilution of 10^3 suggesting significant colicin production.

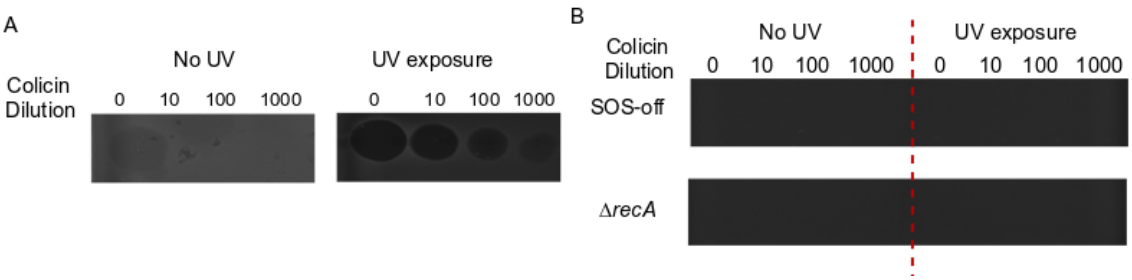


Figure 9: MP1 colicin is active A) inhibition halo of *E. coli* MG1655 by cell lysates of wild-type MP1 either exposed to UV or with no UV exposure B) SOS-off mutant, and *ΔrecA* mutant either exposed to UV or with no UV exposure.

To reinforce SOS regulation on MP1 colicin expression, we employed our previously constructed SOS-off mutant and another MP1 strain, *ΔrecA*. Deleting *recA* prevents LexA cleavage from occurring as the RecA protein is essential for LexA cleavage *in vivo* thus rendering the bacteria unable to activate the SOS response. Utilizing this *ΔrecA* strain is an orthogonal method of turning off the SOS response that should largely phenocopy the results seen with the SOS-off mutant (Mo, 2016). If the two strains provide similar results it would strengthen the conclusion that MP1 colicin is regulated by the SOS response. As such, cell lysates of SOS-off and *ΔrecA* strains were prepared and spot plated as previously described. In the absence of exposure to UV light there were no clear or turbid zones of inhibition seen for either strain (Figure 9B). After UV exposure, there remained no clear or turbid zones of inhibition, suggesting there were undetectable colicin levels. This confirms that the SOS response is required for colicin production. Further, there was no evidence of basal colicin expression in either mutant suggesting that colicin production may be extremely limited in the absence of a

functional SOS response. Collectively, these data demonstrate that MP1 produces an active colicin in the presence of genotoxic stress suggesting that MP1's colicin is important for MP1 during times of stress.

3.3.3 MP1 colicin selectively targets *Enterobacteriaceae* species and enhances MP1 fitness.

Given that MP1 colicin is produced in the presence of DNA damage in an SOS-dependent manner, we next addressed the physiological relevance of MP1's colicin. To investigate the activity spectrum of MP1 colicin, we screened both pathogenic and non-pathogenic *Enterobacteriaceae* species for their sensitivity to MP1 colicin (Table 2). We screened representative *Escherichia coli* strains, which include commensals from group A and B2, an extra-intestinal *E. coli* that is closely related to MP1, intestinal pathogens, and an "atypical" *E. coli* Clade 1 isolate. Further, recent work has suggested that some colicins can kill phylogenetically-distant bacterial species (Riley, 2003) and therefore, we screened *Salmonella* strains with different host reservoirs, a representative *Vibrio cholerae* strain, and a clinical isolate of *Klebsiella pneumonia.* We observed that all strains tested were resistant to the colicin except for *Escherichia coli* Nissle 1917.

Strain	Relevant characteristics	Sensitivity profile	Citation
***Vibrio Cholerae* C6076**	El Tor, O1	Resistant	(Joelsson, 2006)
Klebsiella pneumonia	ST-11 serogroup	Resistant	J. Zhu
***S. typhimurium* 14028s**	Outgroup GC1	Resistant	(Jarvik, 2010)
***S. typhimurium* AJB3**		Resistant	D. Schifferli
***S. Gallinarum* ATCC700623**	Biovar Gallinarum	Resistant	D. Schifferli
***S. Newport* SL254**	S. Newport IIC	Resistant	D. Schifferli
E. coli E2348/69	Group B2, EPEC	Resistant	(Iguchi, 2009)
E. coli CFT073	Group B2, UPEC	Resistant	(Mobley, 1990)
E. coli Nissle 1917	Group B2, Commensal	Sensitive	(Grozdanov, 2004)
E. coli 042	Group D, EAEC	Resistant	(Nataro, 1985)
***E. coli* HS**	Group A, Commensal	Resistant	(Rasko, 2008)
***E. coli* O157:H7 EC4115**	Group B, EHEC	Resistant	(Eppinger, 2011)
MG1655	Group A, Commensal	Sensitive	*E. coli* Genetic Stock Center, CGSC no. 7740
TW10509	*Clade* I, ETEC	Resistant	(Luo, 2011)

Table 2: **Characteristics and sensitivity profile of strains used to test the activity spectrum of MP1 colicin**.

Abbreviations: EPEC, Enteropathogenic *E. coli*, UPEC, Uropathogenic *E. coli*, EAEC, Enteroaggregative *E. coli*, EHEC, Enterohemorragic *E. coli*, ETEC, Enterotoxigenic *E. coli*

We then wanted to understand whether the MP1 colicin is an important component of MP1's ability to compete with other *Enterobacteriaceae*. For this experiment we chose the colicin sensitive strain, Nissle 1917 and *E. coli* HS as our representative resistant strain (Grozdanov, 2004; Rasko, 2008). *E. coli* HS was selected because it is also a commensal. We performed *in vitro* pairwise fitness competitions with MP1. Both the

Nissle 1917 strain and the HS strain are marked with a tetracycline resistance cassette and mCherry under the tight control of a *tet* promoter (Lasaro, 2009). For the competition experiments we utilized a wild-type MP1 derivative containing GFP under the control of the *tet* promoter to allow facile discrimination of the MP1 strain and either mCherry-Nissle 1917 or mCherry-HS. To understand the contribution of MP1 colicin to the fitness of MP1 we constructed a colicin-deficient MP1 strain replacing the colicin protein with a kanamycin-resistant cassette and verified that the colicin-deficient mutant no longer secreted colicin and had similar growth kinetics as the wild-type strain (data not shown) (Murphy, 2003). The colicin-deficient strain was constructed in a wild-type MP1 derivative containing GFP under the control of the *tet* promoter to again allow for facile discrimination of this strain and the mCherry-Nissle 1917 or mCherry-HS strain.

In the case of the colicin resistant strain, HS, no fitness defect was observed in the presence of wild-type MP1 or the colicin-deficient strain for either 24 or 48 h (Figure 10A). However, the wild-type MP1 strain rapidly outcompeted Nissle 1917 with no detectable colonies after 24 h (Figure 10B). In the presence of the colicin-deficient strain, the large fitness defect was abrogated thus suggesting MP1 colicin is negatively affecting the fitness of Nissle 1917. In the presence of the colicin-deficient strain, Nissle 1917 still had a mild fitness defect, suggesting that there are other factors that could enhance MP1 fitness in competition with Nissle 1917. Nevertheless, this data supports the conclusion that colicin production augments the fitness advantage of MP1 over Nissle 1917 and has no effect in the competition with a colicin-resistant strain, HS.

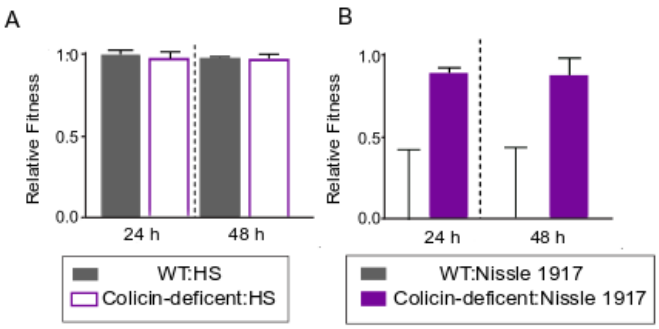


Figure 10: MP1 colicin provides a fitness advantage *in vitro* (A) Relative fitness levels were evaluated in competition experiments between mCherry labeled *E. coli* HS (B) and mCherry labeled *E. coli* Nissle 1917 at 24 h and 48 h. The mean fitness level was calculated from four independent competition experiments. No colonies were detected with Nissle 1917 at 24 h and 48 h, with the top of the error bar representing the limit of detection.

3.3.4 The colicin-deficient strain has equal fitness relative to the colicin-producing strain.

MP1 colicin is active against select *Enterobacteriaceae* and in the presence of MP1 colicin-sensitive *E. coli,* provides MP1 with a fitness advantage over the colicin-sensitive strain. Taken together these data suggest that MP1 colicin may be an important tool for MP1 in order to survive in its natural environmental niche, the mammalian gut. Therefore, we sought to understand the whether MP1 colicin promotes successful colonization of MP1.

MP1 is an ideal model to study the importance of colicin production for commensal colonization because MP1 is a natural *E. coli* mouse isolate and thus can achieve stable colonization in the absence of continuous antibiotic treatment. Most *E. coli* colonization studies enrich for the strain of interest by engineering the strain to be streptomycin resistant and treating the mice with streptomycin for the duration of the experiment (Sweeney, 1996; Meador, 2014). Streptomycin treatment overcomes colonization resistance by eliminating significant numbers of facultative anaerobes

effectively reducing the microbial diversity in the murine gut (Hentges, 1990; Bazett, 2016). In contrast, the MP1 model has a brief streptomycin pre-treatment followed by a 24 h washout period where streptomycin is removed from the water and fresh water is supplied for the remainder of the study. After 5 to 6 days following streptomycin treatment, the normal flora rebounds (Antunes, 2011), allowing us to address whether colicin production impacts colonization in a gut with the associated microbial diversity intact.

First, we wanted to explore whether colicin production was important for MP1 to colonize the murine gut. To do this we utilized our previously-constructed colicin-deficient strain which has a kanamycin resistance marker in place of the colicin gene. In the construction of the colicin-deficient mutant, only the colicin gene was disrupted. The MP1 plasmid encodes not only the colicin, but genomic analysis revealed the presence of lysis and immunity proteins, which is consistent with colicinogenic plasmids (Cascales, 2007). Importantly, the immunity protein confers resistance to the produced colicin. We tested the functionality of the immunity protein in the colicin-deficient mutant and found that indeed the colicin-deficient mutant maintained resistance to the colicin (data not shown). Therefore, using this strain we could specifically interrogate whether the lack of colicin production was important for MP1 colonization.

Taking advantage of the well-established MP1 mouse model, we orally inoculated C57BL/6 male mice with equal mixtures of colicin-deficient mutant *gfp*-marked strain and a wild-type *mCherry*-marked strain. With the aim of understanding colonization dynamics, we quantified the initial inoculum and collected feces both at the beginning of the experiment and continuously throughout the experiment. At each time point, feces were normalized by weight, serially diluted, and plated onto LB agar containing tetracycline which permits selection of the MP1 strains and induces the

expression of either *gfp* or *mCherry* (Figure 11A). We calculated the competitive index (CI) in order to quantify the relative fitness of the colicin-deficient mutant relative to the wild-type strain. CI was calculated by taking the ratio of input CFU counts to output CFU counts. Up to 7 days post-inoculation the was no colonization defect between the wild-type versus the colicin-deficient mutant (Figure 11B, log CI 0.061). By day 28, however there was a mild colonization defect where the wild-type strain outcompeted the colicin-deficient strain by 5-fold (log CI -0.5). CFU counts of each strain reflected the competitive index in that the wild-type strain sustained slightly higher levels then the colicin-deficient strain (Figure 11C). Our data indicates that both strains can stably co-colonize the murine gut, and in this mixed population the inability to produce colicin has minimal impact on colonization.

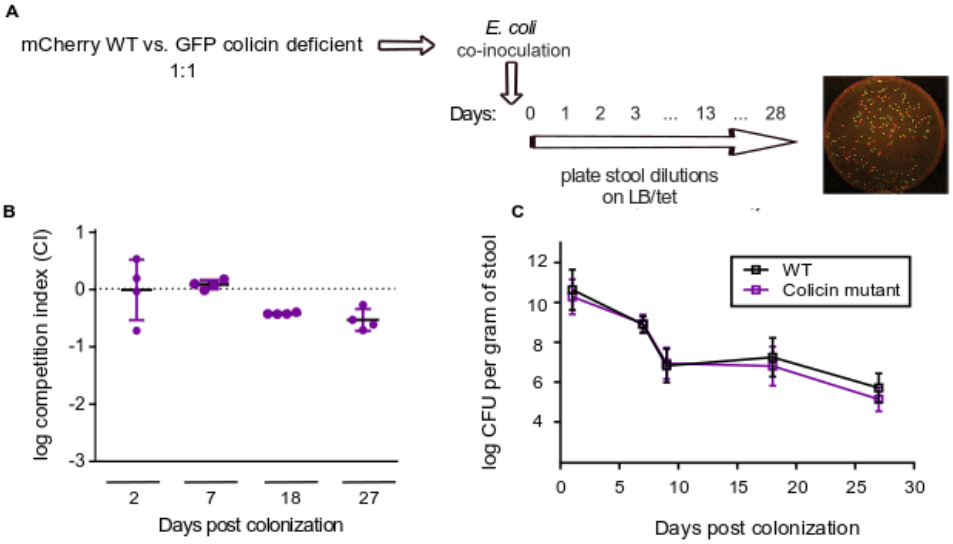


Figure 11: Competition in the adult mouse gut. (A) Schematic of the colonization protocol. Mice were inoculated in a 1:1 ratio of either strain and fecal samples were collected at various days post-inoculation. Fecal samples were serially diluted and plated onto LB/tetracycline plates. A representative plate is shown. (B-C) groups of 6-to-8 week old C57BL/6 mice were co-inoculated. (B) Log competitive index (CI) was calculated by taking the ratio of the output colonies normalized to the input ratio. Each circle represents one specific animal. (C) CFU counts per gram of feces for the competition experiment. Each square represents the mean and standard deviation from all the individual mice. Limit of detection was 10^3.

3.3.5 Sustained colonization does not require colicin production.

Given that previous work has suggested that colicin production may be important for sustained bacterial colonization (Gillor, 2009), we wanted to further investigate the colonization ability of the colicin-deficient strain. Further, since the colicin-deficient strain is also resistant to the colicin, we thought it could be conceivable that the engineered strain was taking advantage of wild-type colicin production. Therefore, we wanted to determine whether the colicin-deficient strain was benefitting from the colicin production of the wild-type strain thus masking any fitness defect.

To address this possibility, we tested the ability of the colicin-deficient mutant to colonize the murine gut independent of the wild-type strain. We orally inoculated C57BL/6 male mice with either the wild-type strain alone or the colicin-deficient strain alone. In both systems, there was no statistical difference between the colonization levels of the colicin deficient mutant and the wild-type strain (Figure 12). The ability of the colicin-deficient strain to stably colonize the murine gut indicates that in the direct competition experiment with the wild type strain, the colicin-deficient strain was not significantly benefiting from colicin production. Taken together our data indicates that in an unperturbed gut environment, sustained colonization does not necessitate colicin production. Our data further suggests that MP1 existing in its natural niche environment may not face bacterial competition that requires colicin production.

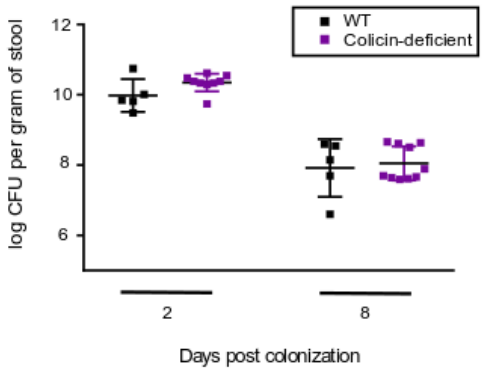


Figure 12: Solo colonization of colicin-deficient strain in the adult mouse gut. Groups of 6-to-8-week-old C57BL/6 male mice were orally inoculated with either the wild-type strain (black squares) or the colicin-deficient strain. On the days indicated, fecal samples were collected and CFU counts were determined Each square represents one mouse and the limit of detection was 10^3.

3.4 Discussion

Colicin production is a generally assumed to be a critical mechanism by which *Enterobacteriaceae* compete with closely-related species during times of stress and scarcity of resources. The abundance and diversity of colicins points to their importance in promoting successful bacteria survival in complex dynamic microbial communities (Riley, 1998). Further, theoretical and *in vitro* studies have proposed that colicin production ensures survival of the producing bacteria (Czaran, 2001; Kerr, 2002). These studies have generated a plethora of models suggesting that colicin production is a critical component for successful bacterial survival and interaction with the natural environment. However, with a few exceptions, they lack direct testing in complex physiological environments.

The mammalian gut is composed of over 10^{13} bacteria and the intimate connection between host health and the microbiota make the gut an ideal setting to test the importance of colicin production in a complex microbial environment. Prior attempts

to address this question utilized mouse-models where the resident facultative anaerobes had been eliminated and *E. coli* strains had been engineered to express a colicin of choice (Kirkup, 2004; Gillor, 2009). Collectively, those studies suggest the importance of colicin production for gut colonization. However, whether a natural colicin-producing *E. coli* commensal requires colicin production for sustained colonization in an unperturbed mammalian gut remained unclear. Towards that end, the aim of this study was to characterize colicin production in the natural *E. coli* MP1, and to directly interrogate the fitness benefit of colicin production for MP1. Importantly, our results indicate that colicin production provides a fitness benefit in the presence of sensitive strains *in vitro*, yet interestingly, in a healthy mouse gut with an intact microbiome, MP1 does not require colicin production for sustained colonization. This is the first report demonstrating that in an unperturbed gut microbiome, colicin production is expendable for colonization of a commensal *E. coli* strain.

Here, we demonstrate that MP1 colicin production is increased in the presence of genotoxic stress in a SOS-dependent manner. The SOS response is a DNA damage repair pathway that is tightly regulated and poised to rapidly respond to genomic stress. Genes regulated by the SOS response are induced in a highly-organized chronological manner where the first genes to be induced are those involved in error-free repair and the last genes to be induced are those involved in error-prone repair (Courcelle, 2001; Friedman, 2005). Error-prone repair reduces genomic integrity and can introduce life-promoting adaptive mutations but can also result in mutations that are deleterious to the cell (Foster, 2007). Consequently, it is hypothesized that induction of error-prone repair is reserved for when there is extensive or sustained DNA damage. MP1 colicin production is induced later in the SOS response suggesting that colicin production is reserved for times of sustained or considerable DNA damage (Butala, 2012). It is

intriguing to consider in this context how colicin production could synergize with the theory of kin selection and cooperation (Wall, 2016). Colicin production results in lysis of the producing cell (Majeed, 2013) thus, in this model, colicin production would occur in cells whose probably of surviving the genomic insult is incredibly low. Colicin release could have two major functions for the clonal population at large; one it could serve as a signal to surrounding clones of genotoxic stress, or two, it could assist in the survival of the population by killing the competitor bacteria.

Previous work in our lab reported that the SOS response is important for sustained colonization of MP1 in a natural gut environment (Samuels, 2019). In that report, an SOS-off mutant had a colonization defect relative to a wild-type strain, and we speculated that the loss of colicin production might contribute to the reduced fitness of the SOS-off mutant. The results presented here suggest that the fitness defect of the SOS-off mutant in the unperturbed gut might be independent of colicin production. It is possible that the genotoxic stress experienced during colonization is not enough to activate late SOS-induced genes but is enough to require activation of early SOS-induced genes. Additionally, the fitness defect of the SOS-off mutant persisted in the setting of DSS-induced gut inflammation. Here, we did not test the role of colicin production during gut inflammation and it is possible that different SOS-controlled effector proteins are responsible for the colonization defect of the SOS-off mutant in different gut environments.

Colicins are potent and specific toxins that result in the rapid elimination of sensitive bacteria (Riley, 2007). The narrow toxin range of colicins supports a model that colicins are important in order to mediate intra-species population dynamics (Riley, 1999). However, there are reports that suggest some colicins have a broader phylogenetic

killing range and consequently could possibly mediate population dynamics on a community level (Riley, 2003). The killing breadth of MP1 colicin was investigated by screening various *E. coli* isolates that are representative of different phylogenetic groups. Among the bacteria screened, we found that MP1 colicin potently inhibited *E. coli* Nissle 1917. MP1 colicin did not inhibit strains closer phylogenetically to MP1 such as the *E. coli* UPEC strain CFT073 and the *E. coli* EPEC strain E2348/69. This was surprising because even though Nissile 1917 and MP1 are both within the B2 phylogenetic group, Nissle 1917 is more distantly related to MP1 than other *E. coli* strains tested. Therefore, from our representative screen of *Enterobacteriaceae* species it seems that MP1 colicin is an example of a colicin that has a broad phylogenetic range and thus could mediate community level population dynamics. It would be interesting to screen more enteric bacteria to investigate whether a pattern of killing emerges. Additionally, Nissle 1917 is an efficient colonizer of the human gut and as such is being used as a commercially available probiotic (Grozdanov, 2004). Interestingly, however, Nissle is a poor colonizer of the murine gut and cannot sustain colonization past 6 days (Lasaro, 2014). MP1 is naturally isolated from the mouse gut and raises the interesting question of whether there is direct competition of Nissle and MP1 for the same resources in the murine gut. If Nissle is in direct competition with MP1 in the murine gut, colicin production by MP1 could be one reason Nissle is not successful in efficiently colonizing the mouse gut.

In *in vitro* competition experiments, MP1 colicin production provided MP1 with a competitive advantage over colicin-sensitive strains suggesting that colicin production for MP1 is important for MP1 to successfully compete with surrounding bacteria. Yet, in a mouse gut with an intact microbiome, sustained colonization did not require colicin

production. In competition with a colicin-deficient but resistant strain, both strains co-existed for the duration of the experiment with only a mild fitness defect. Importantly, we wanted to verify that the colicin-deficient strain was not capitalizing on colicin production of the wild type strain, essentially participating in cheating behavior (Ozkaya, 2017). In this scenario, the colicin-deficient yet resistant strain would be benefiting from colicin production and not paying a cost, thus being able to coexist and masking a fitness defect associated with loss of colicin production. However, during solo colonization, the colicin-deficient mutant was recovered with similar CFU to that of the wild type strain suggesting that the colicin-deficient strain can successfully colonize the murine gut independent of a colicin producing strain.

It is possible that if the duration of the solo colonization experiment had been extended there would have been a reduction in colicin-deficient colony counts. The importance of colicin production for long term colonization has been previously observed and in that experiment the benefit of colicin production was revealed only after ~ 3 months of colonization (Gillor, 2009). However, that experiment was done in the context of an altered microbiome with the mice being continually fed streptomycin for the duration of the experiment. It is entirely possible that in a streptomycin treated mouse, colicin production is critical because of the perturbations to the gut microbiome induced by streptomycin

The results of our mouse competition studies parallel theoretical studies which predict in a mixed population, colicin production will promote a population of bacteria where colicin-sensitive, colicin-resistant, and colicin-producing strains all co-exist, thus enhancing bacterial diversity (Durrett, 1997; Kerr, 2002). In our model, both the colicin-resistant and the colicin-producing strain co-existed throughout the experiment. Our

data, however, deviates from a prior *in vivo* colicin competition study which demonstrated that strain diversity decreased over time (Kirkup, 2004). There are two main explanations for the discrepancy. First, in the previous study, the mice were treated with streptomycin throughout the experiment thus eliminating a significant portion of microbial diversity and subsequently impacting the homeostatic environment of the gut microbiome. Second, our experiments were terminated after 4 weeks. It is conceivable that population dynamics were not captured completely in this time frame and if the duration of the experiment was increased one strain would have eventually dominated.

Overall, the results of our study combined with previous data suggest a model where the fitness afforded by colicin production may depend upon the environmental condition. MP1 is a natural *E. coli* isolate of the murine gut and, by extension, when it is colonizing a healthy mouse gut the environmental stressors might be minimally perturbing. In light of our previous study demonstrating a role for the SOS response during MP1 colonization of the murine gut, it is possible that basal genotoxic stress is present requiring some activation of the SOS response, but the genotoxic stress is not extensive enough to induce colicin production in any appreciable amount. Our model is further supported by a report from Nedialkova et. al. where *Salmonella enterica* serovar Typhimurium strain SL1344 carrying a ColIb colicin gene out-competed a colicin sensitive *E. coli* MG1655 in the inflamed gut (Nedialkova, 2014). Importantly, in the absence of inflammation there was no benefit to colicin production.

With these results in mind, it is interesting to speculate on the microbial equilibrium that may be ongoing even in densely-colonized environments like the mammalian gut. It is reasonable to propose that in the absence of exogenous perturbations competition between bacterial communities is not as fierce as previously imagined, but instead these communities exist in a delicate equilibrium that does not require routine activation of

bacterial defense mechanisms. This report does not address whether colicin production is required when there is direct competition for the niche from a colicin-sensitive strain nor does it address how colicin production alters the fitness of MP1 when there are perturbations to the homeostatic environment. Both of these fundamental questions are being actively explored in our lab. The model used here mimics the microbiome of a healthy mammal living in the wild. In this setting, colicin production is not required for sustained colonization and suggests that maintenance of microbial diversity and fitness in homeostatic environments may rely on alternative mechanism besides colicin production.

3.5 Materials and Methods

3.5.1 Isogenic strain construction. The mCherry-marked MP1 (also known as MP7), *E. coli* Nissle 1917, and *E. coli* HS strains were previously described and used for competition experiments (Lasaro, 2009, 2014). The GFP-marked MP1 strain (also known as MP13) was used for colicin-deficient strain construction and competition experiments (Lasaro, 2014). The colicin-deficient strain was constructed by deleting the colicin gene (colY) via recombineering (Murphy, 2003) and replaced with a kanamycin resistance cassette. The ColY gene is on a plasmid and care was taken to remove the WT plasmid by re-streaking the strain on increasing concentrations of kanamycin. The colonies were screened by PCR for loss of the WT plasmid. The SOS-deficient MP1 strain was described previously and used for the GFP reporter assay (Samuels, 2019). The *⊿recA* MP1 strain was constructed by transduction utilizing a *recA*+ donor strain BW 26547 *recA::kan* Lambda *recA*+. Integration of *recA::kan* was confirmed both by growing the strain in the presence of kanamycin, and by PCR and sequencing.

3.5.2 GFP-reporter assays. Induction of the colicin promoter following DNA damage was monitored by constructing a reporter plasmid containing GFP under the control of the *colicin* promoter. This GFP reporter construct was constructed in a manner similar to the library of GFP reporter plasmids developed by Zaslaver, et. al. (Zaslaver, 2006). To construct this plasmid, we amplified a 450 bp region of the MP1 genome that included 300 bp upstream of the SOS binding boxes of the MP1 colicin gene and 150 bp into the colicin-coding region. This DNA fragment was cloned into the vector pUA66 and the construct was sequenced. Additional reporter plasmids contained GFP under the control of the *recA* promoter or *dinB* promoter as described previously (Culyba, 2018). Briefly, each bacteria strain was transformed with the GFP-reporter plasmids and cultured in minimal media containing 1 X M9 salts (Sigma M6030), 0.4% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.05% Casamino acids, and 30 μg/ml of kanamycin for plasmid maintenance. Overnight cultures were diluted 1:1,000 into fresh media and incubated at 37 ^{O}C shaking until reaching an optical density at 595 nm of ~0.3. 100-μl aliquots were dispensed into 96-well, round-bottom, transparent plates. DNA damage was induced by UV light (50 J/m^2). Plates were incubated at 37^{O}C under agitation and GFP fluorescence (Ex/Em: 485 nm/535 nm) and culture density (OD_{595}) were monitored continuously in 5 min intervals for 3 hr. To prevent evaporation, 50 μl of mineral oil (Sigma) was added to each well. The level of promoter induction was determined by taking the ratio of the fluorescence intensity and the optical density (FI/OD).

3.5.3 Colicin production assays. Colicin lysates were prepared by utilizing a standard colicin production protocol (Riley, 2003). Briefly, strains were grown overnight in LB at 37 ^{O}C shaking. Overnight cultures were diluted 1:100 into fresh LB media and strains grew to an OD_{595} of approximately 0.2-0.3. At this point, 1.0 ml of culture was transferred to an

Eppendorf tube and spun in a microcentrifuge at 9000 rpm for 5 min and resuspended in 0.5 ml of 10 mM $MgSO_4$. Cells were transferred to 24-well plates and exposed to UV light. Following exposure to UV light, 0.25 ml of cells were transferred to 2.0 ml of LB, covered in aluminum foil, and incubated at 37 ^{O}C shaking for 3 hr. Following incubation, 100 μl of chloroform was added, and tubes were transferred to Eppendorf tubes, vortexed for 15 s and spun in a microcentrifuge at 9000 rpm for 10 min. Following centrifugation, the supernatant was transferred to a clean glass tube and stored at 4 ^{O}C. For lysate preparation in the absence of UV light, the steps remained then same except without exposure to UV light.

3.5.4 Colicin screening assays. All strains used in this assay are described in Table 1 Screening assays were prepared by growing each *Enterobacteraceae* strain in LB at 37 ^{O}C, shaking. Indicator lawns for each strain were prepared by adding 50 μL of the cells (~ 1 x 10^9 cells/mL) to 3.5 ml of top-agar, gently mixing, and then pouring onto LB agar plates. After the lawns solidified, 8 μl of lysate was spotted on the lawn in serial dilutions. All indicator strains and lysates were assayed in duplicate.

3.5.5 *In vitro* competition assay. The competition assay was adapted from previously established protocols (Mo, 2016). Briefly, *gfp*-tagged or *mCherry*-tagged strains were grown overnight in LB at 37 ^{O}C shaking. Strains were standardized by optical density, diluted 10^6 -fold and mixed at a 1:1 ratio in 3 ml of LB. The mixture was incubated overnight at 37 ^{O}C shaking for 24 h. At this time point, the overnight mixture was diluted 10^6-fold and inoculated into fresh LB and grown for another 24 h. Culture samples were taken at time zero, 24 h, and 48 h and plated onto LB agar containing 15 μg/ml of tetracycline in order to determine CFU for each strain. Plates were incubated overnight at 37 ^{O}C and imaged using a system that permits the detection of GFP and mCherry

(Siryaporm, 2008). Relative fitness data were calculated by formula described by Lenski et. al. (Lenski, 1991).

3.5.6 Animal experiments. All animal studies were carried out in accordance with guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania. Animal protocols followed the guidelines established within the "Guide for the Care and Use of Laboratory Animals" published by the National Research Council of the National Academies.

Experiments were performed with 6-to-8-week-old C57BL/6 male mice purchased from Charles River Laboratories. All mice were fed standard rodent chow *ad lib* (LabDiet 5001) and had access to fresh water. Each cage contained 4 to 5 mice. The colonization protocol has been described previously (Samuels, 2019), but briefly mice were given 5 g/liter of streptomycin and glucose in their drinking water for 72 h. After 72 h, mice were given fresh water for 24 h prior to oral inoculation with *E. coli* strains and were maintained on fresh water throughout the remainder of the experiments. The inoculum preparation was also described previously (Samuels, 2019), but briefly, cells were prepared by streaking out on LB agar plates and incubating at 37 ^{O}C overnight. The next day, a single colony was picked and grown overnight in LB shaking at 37 ^{O}C. The following day optical density at 595nm was measured and the concentration of cells was calculated. Cells were spun down at *3,800 x g* at 4 ^{O}C and resuspended in cold PBS. Cells were washed twice with PBS and resuspended to a final cell density of ~ 10^{10} to 10^{11} cells/ml. For the competition experiment, cells suspensions were mixed at a 1:1 ratio and mice were orally inoculated with ~100 μl. For the solo colonization experiment ~100 μl of each strain was given to each mouse.

To determine the colony counts, at each time point 3 to 4 pellets of poop was obtained from each mouse. Fecal samples were weighed and resuspended in PBS to reach a final concentration of 0.5 g of feces per 1.0 ml of PBS. The samples were vortexed and incubated at 4 ^{O}C for roughly 1 h. The samples were serially diluted in PBS and plated on LB agar plates containing 15 μg/ml tetracycline. Fluorescence images of the plates were obtained as described previously. The competitive index (CI) was determined as [(mCherry fluorescent CFU)/(GFP fluorescent CFU)]/[(input mCherry CFU/input GFP CFU)], where the initial inoculum represents input CFU.

Chapter 4: Diversity Among Intrinsic LexA Self-Cleavage Rates Across Bacterial Species Reveal Conservation of the LexA:RecA* Interaction

This chapter is the basis of a future manuscript. Our ongoing efforts to expand on this chapter for the manuscript are discussed more extensively in Chapter 5.

4.1 Abstract

The bacterial SOS response is a highly conserved DNA repair pathway that is activated in the presence of DNA damage. The SOS response is regulated by LexA, a transcriptional repressor-protease that binds to DNA regions upstream of SOS-controlled genes preventing their expression. Upon DNA damage, single-stranded DNA forms and RecA, the sensor of the SOS response, filaments along these DNA fragments. The activated RecA filaments bind to LexA and promote a LexA self-cleavage reaction. Cleaved LexA cannot bind DNA and consequently the SOS response is activated. The LexA self-cleavage rate is the critical step in activation of the SOS response and it is unknown whether the rates of LexA self-cleavage are conserved across bacterial species. Here, we systematically characterize the self-cleavage rate of LexA proteins from a diverse set of bacterial species: *Escherichia coli* ($LexA_{Ec}$), *Bacillus subtills* ($LexA_{Bs}$), *Clostridium difficile* ($LexA_{Cd}$), *Pseudomonas aeruginosa* ($LexA_{Pa}$), *Mycobacterium tuberculosis* ($LexA_{Tb}$), and *Vibrio cholerae* ($LexA_{Vc}$). Rates were determined at alkaline pH in the absence of RecA, as well as at physiological pH in the presence of RecA. We find that under alkaline conditions, the cleavage rates were dramatically different with $LexA_{Vc}$, $LexA_{Pa}$, and $LexA_{Ec}$ having similar cleavage rates, $LexA_{Bs}$ have a reduced cleavage rate, and $LexA_{Tb}$ and $LexA_{Cd}$ not having any detectable cleavage. However, in the presence of *E. coli* RecA* all LexA proteins undergo the LexA self-cleavage reaction suggesting that RecA* acts to enhance LexA self-cleavage independent of the inherent LexA self-cleavage rate. This study reveals diversity in the

intrinsic self-cleavage rate of LexA and provides the critical first step towards understanding SOS regulation across bacterial species.

4.2 Introduction

Bacteria can rapidly and effectively adapt to environmental insults making them incredibly successful organisms. The ability of bacteria to effectively respond to environmental challenges is partly due to their vast array of stress response pathways. Stress response pathways are generally composed of a sensor protein and an effector protein which together modulate downstream expression of genes aimed at promoting bacterial survival. The SOS response is an example of a highly conserved stress response pathway that is activated in the presence of DNA damage. The SOS response acts to minimize the lethal consequences of DNA damage and facilitate bacterial survival and adaptation through the induction of SOS regulated genes.

DNA damage is an abundant environmental insult and repairing DNA damage is essential for continued bacterial survival. As such, the SOS response is conserved across nearly all Gram-positive, Gram-negative, and Mycobacterial species (Erill, 2007). In all bacterial species the SOS response is tightly regulated by two highly conserved proteins, LexA, the repressor of the SOS response, and RecA, the sensor of DNA damage. LexA is a dual function repressor-protease that consists of two domains, an N-terminal DNA binding domain and a C-terminal catalytic domain. In the absence of DNA damage, LexA binds to the promoter region of SOS controlled genes and represses their activation (Butala, 2009). When DNA damage occurs, regions of single-stranded DNA (ssDNA) are exposed either as a direct result of the DNA damage or indirectly due to stalling of replication forks around DNA lesions. The ssDNA is recognized by RecA which polymerizes around the ssDNA in an ATP-dependent fashion forming the

activated filament, known as activated RecA (RecA*) (Cox, 2007). This active RecA* filament binds to LexA and promotes a self-cleavage reaction essentially separating the LexA C-terminal domain from the N-terminal domain. Cleaved LexA can no longer effectively bind to DNA, relieving repression of the SOS regulated genes in a coordinated fashion (Culyba, 2018; Courcelle, 2001).

Primarily, the SOS regulon functions to repair damaged DNA and, with a few exceptions, this repair can occur with both high fidelity or low fidelity mechanisms. The SOS genes that result in high fidelity repair include effectors such as nucleotide excision repair enzymes that function to repair pyrimidine dimers (Easto, 1983; Erill, 2007). If the DNA damage is persistent or extensive, low fidelity repair is also observed, predominately occurring through the actions of error-prone polymerases which replicate over damaged DNA with an error rate 1000-fold higher than that of high-fidelity polymerases (Tompkins, 2003; Galhardo, 2009). Importantly, reduced fidelity repair results in a transient hypermutator phenotype that introduces mutations throughout the genome. From a clinical perspective, this enhanced mutagenesis is particularly significant, as SOS activation has been linked to heightened bacterial adaptation and acquired antibiotic resistance (Mo, 2016; Cirz, 2006). While repair mechanisms are a commonality across species, not all SOS-controlled genes are dedicated to genomic repair, an observation that reveals functional divergence in the SOS regulon across different bacteria species. To highlight a few examples the SOS response regulates virulence factor synthesis and fibronectin binding protein in *Staphylococcus aureus* (Bisognano, 2004; Úbeda, 2005), biofilm formation in *Pseudomonas aeruginosa, Clostridium difficile,* and *Listeria monocytogenes* (Gotoh, 2010; van der Veen., 2010; Walter, 2015) and type III secretion in enteropathogenic *E. coli* (Mellies, 2007). Further, the SOS response can regulate mobile genetic elements such as integrons, integrating

conjugative elements, and pathogenicity islands (Beaber, 2004; Maiques, 2006; Guerin, 2009; Krin, 2014). Taken together, the SOS response plays a critical role in bacteria evolvability and in some bacteria species, virulence.

Importantly, the expression of SOS controlled gene is directly related to the LexA cleavage event. As mentioned earlier, LexA protein has two domains a DNA binding domain (N-terminal) and a protease domain (C-terminal) which are connected to each other by a flexible linker. The two domains largely function independent of each other with the chemistry of the cleavage reaction being localized to the C-terminal domain. LexA is part of a family of enzymes characterized by a conserved serine-lysine catalytic dyad that cleavages between an Ala-Gly sequence within the protease domain (Luo, 2001). The cleavage site region consists of a flexible loop of ~16 amino acids containing the critical scissile Ala-Gly bond that is cleaved separating the C-terminal domain from the N-terminal domain (Luo, 2001). Crystal structures of LexA revealed that the cleavage site loop can adopt two distinct conformations, a cleavable and a non-cleavable conformation. In the cleavable conformation the loop is located adjacent to the serine-lysine dyad in the binding pocket and in the non-cleavable state, the loop is 20Å away from the binding pocket (Luo, 2001). In the cleavable conformation, the cleavage loop flips into a hydrophobic binding pocket that contains the catalytic serine and lysine and it is thought chemical interactions here stabilize the loop enabling the cleavage chemistry to occur.

The LexA proteins studied thus far have the unique property of being able to self-cleave under alkaline conditions independent of RecA*, however this self-induced cleavage activity is not thought to occur *in vivo* in the absence of RecA* (Schuldiner , 1986; Dri, 1994). Nevertheless, LexA self-cleavage in alkaline conditions has been a powerful tool to address the molecular mechanism regulating the intrinsic self-cleavage

activity of LexA. To obtain a detailed structure-function analysis of LexA self-cleavage activity, our lab previously performed extensive amino acid profiling of the cleavage site loop of LexA from *P. aeruginosa* and determined cleavage profiles for each position (Mo, 2014). In this study, mutations were discovered that either increased or decreased the intrinsic alkaline-mediated rate of LexA self-cleavage, and these variants maintained parallel alterations to their cleavage rates with RecA*. More globally across species, however, it is unknown if RecA*-mediated and alkaline-mediated cleavage parallel one another. In a follow-up study we determined that varying LexA cleavage rates in *E. coli* had two important physiologic consequences (Mo, 2016). First, LexA cleavage rates correlated positively with the extent of SOS activation and gene induction. Second, we observed that wild-type *E. coli* was better at surviving a genotoxic insult then *E. coli* strains harboring either LexA cleavage variant in place of the wild-type LexA protein. From this, we made the provocative conclusion that LexA cleavage rate may have been evolutionarily optimized to tune regulation of the SOS response. Although the LexA protein is highly conserved, it is not clear whether there are differences in LexA cleavage rate across bacterial species, and thus, by extension, different rates of SOS activation.

LexA exists within a superfamily of LexA-like proteins including phage λ CL repressor which undergoes a similar cleavage reaction as LexA mediating phage transformation to a lytic cycle (Little, 1984). However, the rate of cleavage is significantly slower than LexA and it is hypothesized to be another layer of regulation in order to delay the phage's entry into the lytic cycle until the host experiences elevated levels of genotoxic stress (Mustard, 2000; Kim, 1993). The cleavage characteristics of phage λ CL repressor help to support a hypothesis that LexA cleavage rates may vary across bacterial species due to evolutionary fine tuning of the SOS response. However, there

has been no systemic characterization of LexA cleavage rates from phylogenetically distinct bacterial species to help inform whether LexA cleavage rates are conserved.

To address the nature of conserved versus divergent features of LexA across species, we biochemically characterized the cleavage activity of six different LexA proteins from phylogenetically diverse bacterial species. We expressed and purified each protein in parallel and methodically characterized each LexA proteins' cleavage rate in both conditions of alkaline pH and in the presence of *E. coli* RecA*. In this study, we reveal that LexA self-cleavage in the presence of alkaline pH is not a conserved feature across LexA proteins from different species. Supporting the theory of evolutionary fine tuning, we further demonstrate that in the presence of *E. coli* RecA* all LexA proteins, even those unable to undergo alkaline-induced cleavage, can undergo induced cleavage, albeit with varying cleavage efficiencies. Our work provides the necessary framework to probing the similarities and differences in the rate of SOS gene induction across bacterial species and the molecular conservation of the LexA:RecA interface. Further our work could provide a rational framework for designing broadly-active inhibitors that target conserved aspects of LexA to suppress the SOS response and antagonize bacterial adaptation and virulence.

4.3 Results

4.3.1 Sequence identity and similarity between LexA of multiple species

The LexA protein is highly conserved across all bacterial species with each LexA protein having the archetypal structure of an N-terminal DNA-binding domain, a C-terminal protease domain, and a flexible linker connecting the two domains. In order to probe whether LexA proteins from diverse bacteria species have different rates of self-cleavage, we decided to profile a sub-set of LexA proteins from each class of bacteria:

Gram-positive, Gram-negative, and Mycobacterium. When considering which LexA proteins to investigate we chose organisms that had a previously characterized SOS response and had diverse SOS regulons. With this in mind, we chose to investigate the LexA proteins from the following bacteria species: *Clostridium difficile* ($LexA_{Cd}$), *Bacillus subtilis* ($LexA_{Bs}$), *E. coli* ($LexA_{Ec}$), *Pseudomonas aeruginosa* ($LexA_{Pa}$), *Mycobacterium tuberculosis* ($LexA_{Tb}$), *Vibrio cholera* ($LexA_{Vc}$) (Love, 1986; Courcelle, 2001; Cirz, 2006; Smollett, 2012; Walter, 2014). $LexA_{Ec}$ and $LexA_{Pa}$ were included because they have been previously studied and thus could serve as a benchmark in which to compare the other proteins. After selecting our LexA proteins of interest we performed an amino acid sequence alignment of the C-terminal domain (Figure 13 A-B). We focused on the C-terminal domain because we are interested in the self-cleavage properties of LexA. From the sequence alignment a few observations can be made. As expected, the serine/lysine catalytic dyad and the target Ala-Gly scissile bond, were conserved (Slilaty, 1987). Focusing specifically on regions adjacent to the scissile bond (Ala-Gly P1-P1'), we noted the following: P5 is a conserved glycine across all species; P4 is an arginine in all LexA proteins except $LexA_{Bs}$ and $LexA_{Cd}$ where this residue is a lysine and glutamine, respectively; P3 is a valine in all LexA proteins except $LexA_{Tb}$ and $LexA_{Cd}$ where this residue is an isovaline; P2 is an alanine in all LexA proteins except for $LexA_{Cd}$ and $LexA_{Bs}$ where this residue is a threonine; and P2' is variable across all LexA proteins.

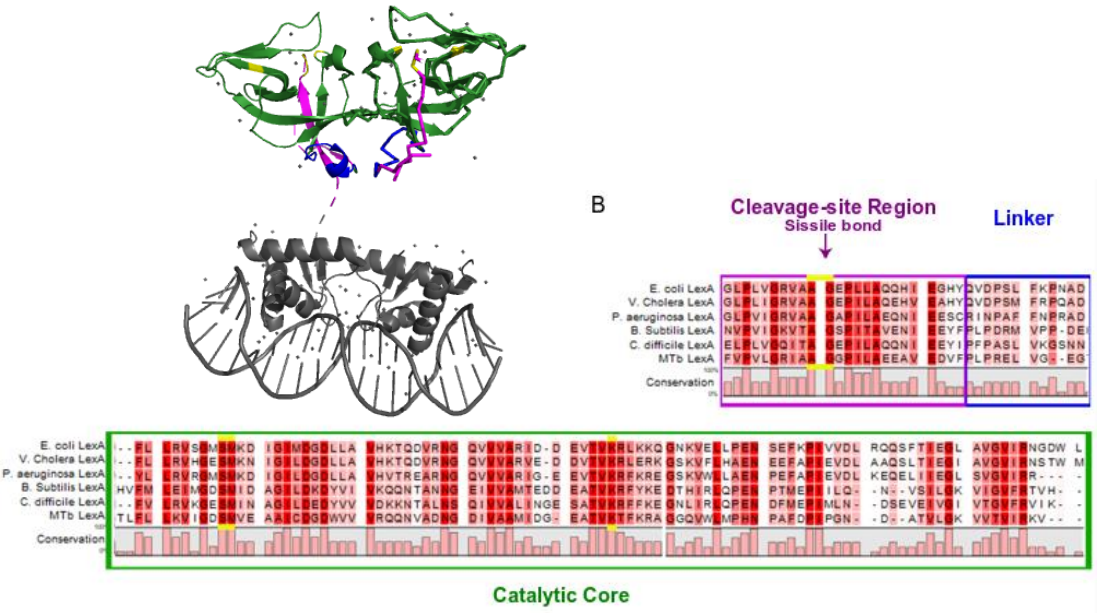


Figure 13: Crystal structure of *E. coli* LexA (PDB 3JSO) dimer (Zhang, 2010) **and LexA protein alignment.** (A) *E. coli* LexA crystalized as a dimer. The N-terminal domain is shaded gray and the C-terminal domain is highlighted by purple, blue, and green. The purple color represents the cleavage-site region which harbors the critical scissle bond. The blue color represents the linker region and the green color represents the catalytic core that contains the serine nucleophile and critical lysine residue. The scissle bond and catalytic serine and lysine residues are highlighted with yellow. These colors match the boxed regions on the LexA protein alignment. (B) The protein alignment of the C-terminal domains for the LexA proteins in this study is shown.

4.3.2 The intrinsic self-cleavage properties of LexA are highly variable

Having selected the LexA proteins of interest, we next proceeded to clone each into identical construct scaffolds. We then expressed and purified the proteins in parallel using recombinant *E. coli*. All proteins were purified by Ni-NTA his-tag affinity chromatography and their purity was determined by SDS-PAGE gel. Throughout the purification there was no noticeable differences in the purification process among the different LexA proteins and each protein ran with the expected molecular-weight on an SDS-PAGE gel.

In our quest to characterize the intrinsic self-cleavage rate of each LexA protein, we began by first investigating their ability to self-cleave under a gradient of pH conditions. Prior work has indicated that alkaline induced cleavage likely reflects deprotonation of the conserved active site Lysine (K156 in *E. coli*) that engages the Serine to make it an effective nucleophile (Slilaty, 1987). Under varied pH gradient in prior work, both $LexA_{Ec}$ and $LexA_{Pa}$ display rapid self-cleavage rates at a pH greater than 9.5 and minimal self-cleavage under more acidic pH conditions (Little, 1984). To assess our broader panel of LexA proteins, we first examined self-cleavage under a pH gradient from 6.6-11.3 (Fig. 14A). For all proteins the cleavage reaction was performed at 37 ºC and initiated by the addition of cleavage buffer (see materials and methods). The cleavage reaction was quenched after 3 h and the reaction was analyzed by SDS-PAGE gel. Cleavage was determined by a loss of the full length LexA protein and the appearance of fragments representing the C-terminal domain and the N-terminal domain.

The pH profile of $LexA_{Ec}$ suggests a model where cleavage requires a pKa of about 9.1 (Slilaty, 1987), yet across our LexA proteins, the pH profile was highly variable. We found that $LexA_{Cd}$, $LexA_{Tb}$, $LexA_{Bs}$ had dramatically different pH profiles relative to $LexA_{Ec}$ with $LexA_{Cd}$ and $LexA_{Tb}$ having no detectable cleavage at any pH tested. $LexA_{Pa}$ and $LexA_{Vc}$ had a similar pH profile to $LexA_{Ec}$ with the pKa of 9.1, and 8.9, respectively. $LexA_{Bs}$, while still having a different pH profile compared to $LexA_{Ec}$, did cleaved in high pH conditions with a pKa of 10.1 (Figure 14B).

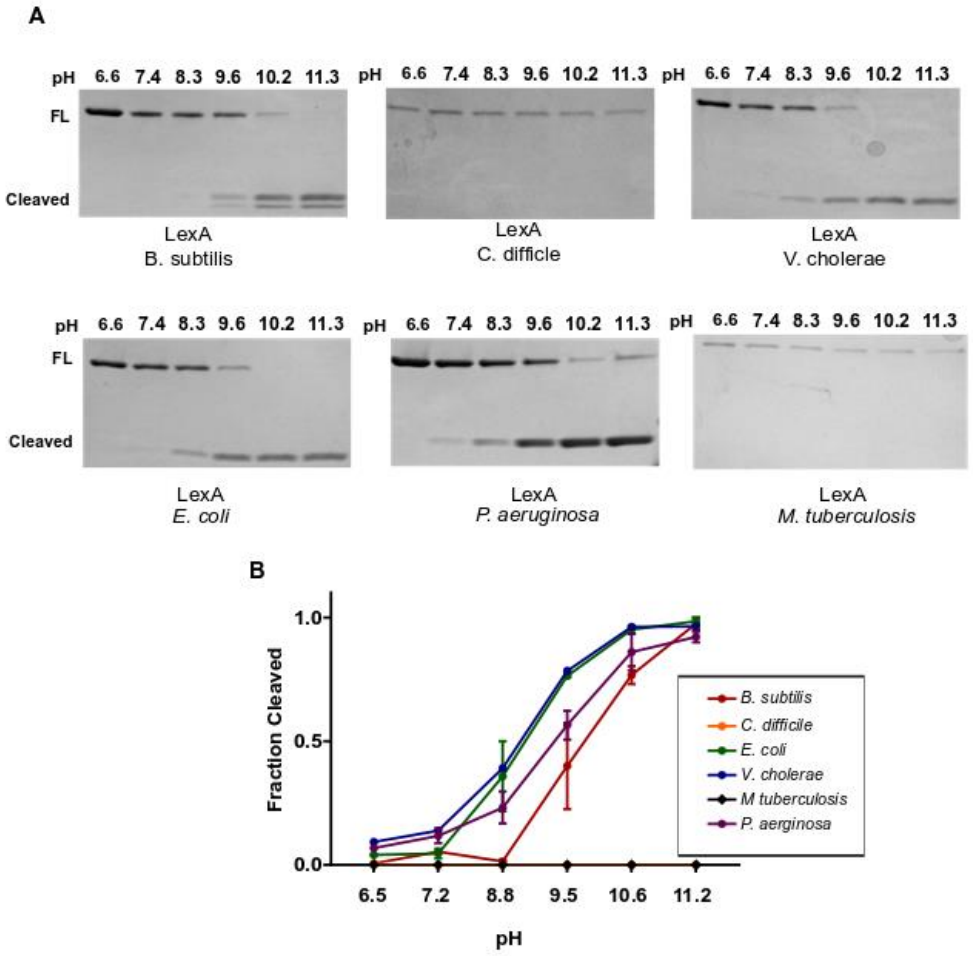


Figure 14: pH profiles of LexA proteins in the absence of RecA* (A) Representative gels demonstrating the pH profile of each LexA protein tested. Proteins were incubated in the respective pH conditions and quenched after 3 hr. (B) Cleavage kinetics for the pH profiles. The fraction cleaved was plotted as a function of pH. The pKa was determined using the program Igor Pro.

For the proteins that cleaved at alkaline pH, we performed a more detailed analysis of their self-cleavage properties in order to determine cleavage rates. Selecting a pH of 10.6 we performed a time course reaction by removing an aliquot of each reaction at various time points and quenching in 2x BME-containing Laemmli buffer (Figure 15A). The cleavage reactions were again analyzed by SDS-PAGE gel. To determine the cleavage rates, we measured the fraction of cleavage product over time

relative to the starting product (Figure 15B). For each LexA protein the half-life and cleavage rates were calculated (Table 3). $LexA_{Ec}$ and $LexA_{Pa}$ the half-life for each protein were 12 min and 25 min, respectively. The half-life of $LexA_{Vc}$ was roughly 17 mins which is similar to both $LexA_{Ec}$ and $LexA_{Pa}$. However, $LexA_{Bs}$ deviated significantly from the other LexA proteins having a half-life of 36 min. In the context of the minimal cleavage of $LexA_{Cd}$ and $LexA_{Tb}$, the $LexA_{Bs}$ result indicates that rapid alkaline-induced cleavage may be an intrinsic feature more closely aligned with Gram-negative LexA variants only. Taken together, these results suggest that the ability of LexA proteins to cleave under alkaline conditions is not a universal property of all LexA proteins. Further, for LexA proteins that do cleave under alkaline conditions, the rates of self-cleavage are highly variable.

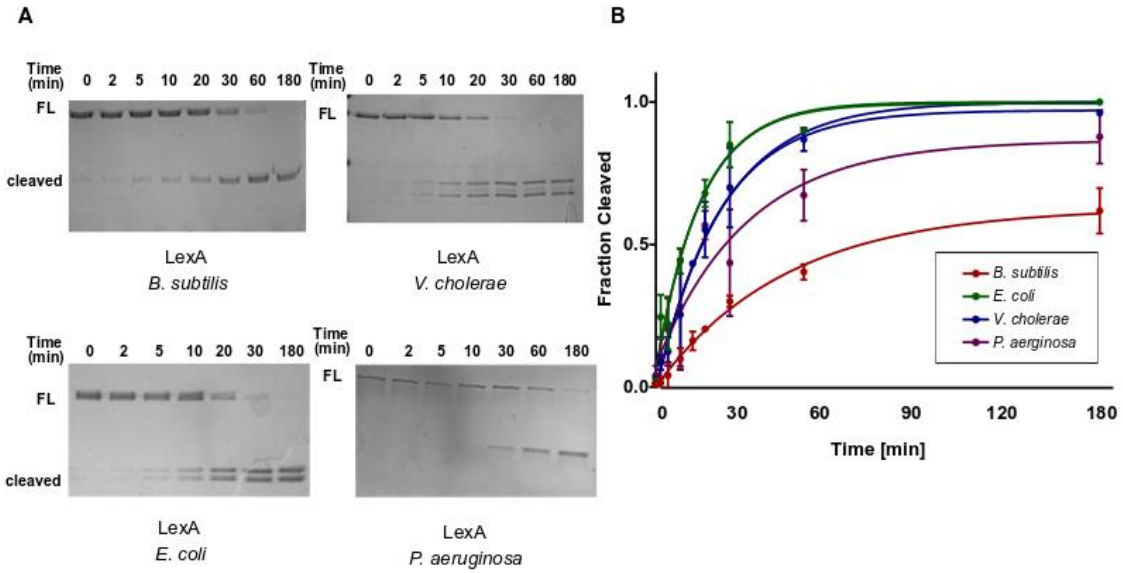


Figure 15: Alkaline cleavage profiles of LexA proteins. (A) Representative time-dependent cleavage profiles of $LexA_{Bs}$, $LexA_{Vc}$, $LexA_{Ec}$, and $LexA_{Pa}$. Proteins were incubated at pH 10.6, and quenched at the given time points, and visualized using SDS-PAGE. (B) Cleavage kinetics of the different LexA proteins. Fraction cleaved was fit as a function of time according to first-order kinetics. Experiments were done in triplicate.

LexA protein	Rate, *k* (x 10^{-5} s^{-1})	Half-life (min)
B. subtilis	31 ± 3.3	36.6
E. coli	92 ± 12	12.5
V. cholerae	66 ± 9.8	17.5
P. aeruginosa	45 ± 10	25.4

Table 3: Rate and half-life values for cleavage of LexA proteins at pH 10.6. Rate and half-life values were calculated in Prism according to first-order kinetics.

4.3.3 *E. coli* RecA* stimulates cleavage activity

In prior studies where LexA cleavage rates were increased or reduced due to amino acid substitutions, the corresponding RecA* mediated cleavage rates paralleled the altered rates (Smith, 1991; Mo, 2014). Therefore, we were curious as to whether this pattern would hold true with our LexA proteins from different species. For the purpose of this study, we chose to explore the LexA cleavage properties of each LexA protein in the presence of *E. coli* RecA*. We chose *E. coli* RecA for two main reasons. One, we wanted to compare RecA*-mediated cleavage with as few of confounders as possible. *E. coli* RecA has been extensively characterized; the purification and RecA activation protocol having been optimized both in the literature and in our lab. Using *E. coli* RecA allows us to compare the cleavage efficiencies of each LexA protein in a setting where the RecA protein is not a variable. Second, reports in the literature suggests *E. coli* RecA* protein can mediate cleavage of LexA proteins from different species (Winterling, 1997; Ambur, 2009; Stohl, 2011; Mo, 2014; Walter, 2014). We theorized that if *E. coli* RecA* induces cleavage of multiple LexA species this would suggest that even though the intrinsic self-cleavage properties of LexA are not conserved, the mechanism of RecA*-induced LexA self-cleavage event may be conserved.

We activated *E. coli* RecA using the standard protocol (see materials and methods). The cleavage reactions were initiated by adding each LexA protein to

previously activated *E. coli* RecA*. For each LexA protein we monitored the LexA cleavage activity over time by removing an aliquot at set time points and quenching the reaction using 2x-BME-containing Laemmli buffer. Each reaction was analyzed by SDS-PAGE gel and stained with Coomassie (Figure 16A). The amount of cleavage was quantified as previously described. Significantly, in this experiment, we found that *E. coli* RecA* promoted LexA self-cleavage of all the LexA proteins tested (Figure 16B).

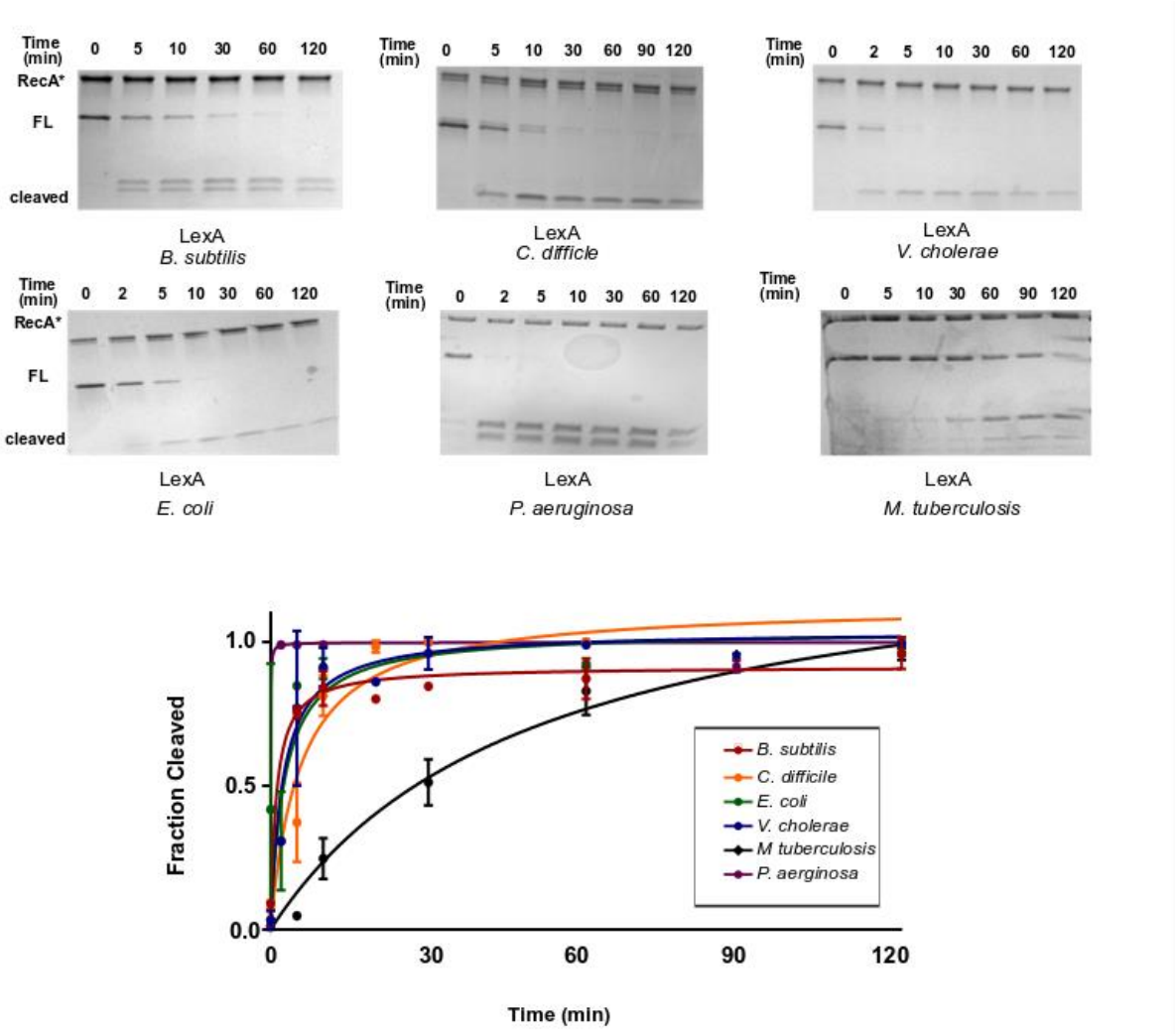


Figure 16: *E. coli* RecA*-mediated cleavage of LexA proteins. (A) Representative time-dependent cleavage profiles of $LexA_{Bs}$, $LexA_{Cd}$, $LexA_{Ec}$, $LexA_{Vc}$, $LexA_{Tb}$, and $LexA_{Pa}$ in the presence of RecA*. Proteins were incubated at 37 ºC, quenched at the given time points, and visualized using 15% SDS-PAGE. (B) Cleavage kinetics of the

different LexA proteins. Fraction cleaved was monitored over time and quantified as described in the text.

$LexA_{Pa}$ and $LexA_{Vc}$ cleaved rapidly in the presence of *E. coli* RecA* with $LexA_{Pa}$ being completely cleaved within 2 mins and $LexA_{Vc}$ being over 90% cleaved by 5 min. $LexA_{Bs}$ cleavage was enhanced in the presence of *E. coli* RecA* with over 50% of $LexA_{Bs}$ being cleaved by 10 min. Although $LexA_{Cd}$ and $LexA_{Tb}$ did not cleave in alkaline conditions, in the presence of *E.coli* RecA* both proteins cleaved. Here, $LexA_{Cd}$ was fully cleaved by 30 min and $LexA_{Tb}$ was greater than ~75% cleaved by 2 hr. These results begin to support a model in which the interaction between LexA and RecA* is highly conserved such that *E. coli* RecA* can promote the self-cleavage event of LexA proteins from a diverse range of bacterial species. Further, our results demonstrate that the ability of RecA* to act on LexA proteins seems to be independent of whether the protein is capable of cleavage under alkaline pH.

4.4 Discussion

The widespread distribution of the SOS response across the prokaryotic kingdom suggests that it is a critically important pathway for promoting bacteria survival during periods of genotoxic stress (Erill, 2007). Despite the ubiquity of the SOS response and its key regulators, LexA and RecA, the SOS regulon varies significantly among bacterial species which has led to the theory that the SOS response may have evolved to fit the needs of different bacteria species (Erill, , 2007; Simmons, 2008; Baharoglu, 2014). SOS gene induction is directly related to the cleavage activity of LexA. If LexA does not cleave, there is no activation of the SOS response (Slilaty, 1987; Mo, 2016). Further, point mutations in the cleavage region of *E. coli* LexA can generate LexA cleavage variants that either cleave slower or faster relative to wild-type *E. coli* LexA protein.

When these cleavage variants are introduced into *E. coli,* there is a progression of SOS activation as a function of LexA cleavage rate (M., 2016). This finding raised the thought-provoking question of whether different LexA cleavage rates naturally existed within LexA proteins from different bacterial species. In this study we set out to address this fundamental question by systematically defining the self-cleavage rates of LexA proteins from a broad range of bacteria species. Our results demonstrate that LexA self-cleavage properties are variable in both alkaline pH and in the presence of RecA*. Interestingly, RecA* promoted the self-cleavage property of all LexA proteins tested irrespective of whether the protein had cleavage activity under alkaline pH.

This study represents the first systematic biochemical characterization of LexA proteins from diverse bacterial species. Our studies reveal that the intrinsic cleavage properties of LexA may not be conserved and conclusions drawn from biochemical studies of *E. coli* and *P. aeruginosa* LexA may not translate to LexA proteins of other bacterial species. That said, from the LexA proteins investigated in this study, no clear pattern or clustering emerged regarding LexA cleavage rates and class of bacteria studied. The LexA proteins from Gram-negative bacteria had similar self-cleavage properties, but the LexA proteins from Gram-positive bacteria did not follow the same trend where $LexA_{Bs}$ cleaved under alkaline pH but $LexA_{Cd}$ did not. Our study would further benefit from increasing the number of LexA proteins investigated. We propose that by characterizing more LexA proteins across the prokaryotic kingdom, a pattern may start to emerge where cleavage rates are reflective of clear phylogenetic differences.

The results of this study allow us to start addressing whether there are specific residues that may be important for the differences in LexA self-cleavage rates under alkaline pH. When the $LexA_{Ec}$ crystal structure was solved, the authors speculated that there could be “fine-tuning” of self-cleavage rates due to specific mutations within the

cleavage site region and/or the hydrophobic surface (Luo, 2001). Previous work performing exhaustive mutagenesis on the cleavage site region in *P. aeruginosa* LexA further supported this speculation because amino acid substitutions were generated that increased or decreased the rate of LexA cleavage relative to the wild-type protein (Mo, 2014). We can use the results presented in that study to help decipher amino acid substitutions near the scissile bond that may be contributing to the altered cleavage rate of the different LexA proteins. $LexA_{Pa}$ has an alanine in position P2 which is conserved in all LexA proteins tested but in $LexA_{Bs}$ and $LexA_{Cd}$ a threonine is substituted. When the alanine in P2 was substituted for a threonine in $LexA_{Pa}$, this variant had a 30-fold reduction in cleavage rate under alkaline pH. The authors speculated amino acids larger than an alanine in this position could increase steric clash of the cleavage site region. In $LexA_{Bs}$ there is a 5-fold difference in cleavage between $LexA_{Pa}$ and $LexA_{Bs}$ and while $LexA_{Cd}$ did not cleave under alkaline pH, cleavage was still observed in the threonine-substituted $LexA_{Pa}$. So, while the amino acid substation at the P2 position may play a role in the differing cleavage activities, based on these results it is not the only mechanism.

Applying this analysis to the P3 position we come to a similar conclusion. Here, $LexA_{Pa}$ has a valine in position P3 which is conserved in all LexA proteins investigated except for $LexA_{Tb}$ and $LexA_{Cd}$ which both have an isovaline. When P3 was substituted for an isovaline in $LexA_{Pa}$, the authors recorded a 3-fold reduction in cleavage under alkaline pH. In our experiments $LexA_{Tb}$ and $LexA_{Cd}$ did not have any cleavage activity under alkaline pH thus an isovaline at this position is not the only explanation for reduced cleavage of these proteins. While mutations in these sites may be contributing to the altered cleavage activity, they cannot account for the extent of altered activity. $LexA_{Tb}$ and $LexA_{Cd}$ did not just have a 3-fold reduction in cleavage under alkaline pH,

they failed to cleave within 3 hr. Taken together, this suggests that is it probably a combination of residues that have evolved to alter the cleavage rate in such a way as to either stabilize the non-cleavable form, destabilize the cleavable form.

In solving the *E. coli* LexA crystal structure the authors captured the LexA protein in two-distinct conformations, a non-cleavable and a cleavable form. In this model the cleavage-site region is the only portion of the C-terminal domain that is mobile (Roland, 1992; Luo, 2001). It has been hypothesized that at neutral pH LexA exists largely in the non-cleavable form, but alkaline pH promotes a shift in the cleavage site region towards the more favorable cleavable form. Even though the crystal structures of $LexA_{Ec}$ captured these two forms of LexA, this is no clear biophysical evidence of a dynamic self-cleavage region (Luo, 2001; Zhang, 2010). The reduced self-cleavage activity of $LexA_{Tb}$, $LexA_{Cd}$, and $LexA_{Bs}$ could support a model where these two conformations exist and suggest the possibly that some LexA proteins may favor an equilibrium that strongly supports the non-cleavable form. However, this is purely speculative and there may be other mechanisms that impact the self-cleavage activity of LexA proteins under alkaline pH. Interestingly, a newly published report suggests $LexA_{Tb}$ may exist in a tetrameric form (Chandran, 2019). It is possible that this may influence the self-cleavage properties of $LexA_{Tb.}$

Here we report that *E. coli* RecA* can promote the self-cleavage activity of all LexA proteins tested regardless of each proteins ability to cleave under alkaline pH. In our studies, neither $LexA_{Tb}$ or $LexA_{Cd}$ cleaved under alkaline conditions, but cleaved in the presence of *E. coli* RecA*. This result confirms the role of RecA* as a catalyst for the LexA self-cleavage reaction (Giese, 2008) and supports a model where the molecular interaction between LexA with RecA* are evolutionarily conserved. If there was no conservation between the molecular interactions of LexA and RecA* we would have

predicted that $LexA_{Tb}$ and $LexA_{Cd}$ self-cleavage activity would have been similar to conditions under alkaline pH. Our data is consistent with the suggestion that RecA* helps to stabilize a cleavage competent form of LexA, but it remains unclear whether RecA* does this through an active mechanism or through an allosteric interaction. As discussed on future directions, this study could be further enhanced by the analysis of RecA constructs from multiple species, rather than *E. coli* RecA only. Such an analysis can inform how the RecA*: LexA interface may have evolved across the phylogenetic tree and provide insights into the conserved features which might be suitable for inhibition.

In this study, we observed different rates of *E. coli* RecA*-mediated cleavage for all LexA proteins tested. We suggest that there are two predominant explanations for this observation. One, it is possible that the differing cleavage rates are a consequence of the different intrinsic self-cleavage rates of each LexA proteins. Second, it is possible that different LexA proteins have varying affinities for *E. coli* RecA* and the differences in affinities is what is causing the variation in *E. coli* RecA* mediated cleavage rate. To address the underlying mechanisms that alters RecA*-mediated cleavage rates, we have initiated and are continuing to characterize the molecular interaction of *E. coli* RecA* with each LexA protein using two complementary approaches: analysis of detailed kinetics of RecA*-mediated cleavage of radiolabeled-LexA under conditions of limiting RecA* and direct measurement of LexA affinity for RecA*. By radiolabeling each LexA protein we can quantitatively characterize initial cleavage rates of each LexA protein and determine the k_{cat} and K_M of each interaction, which would provide insight into the overall kinetics of the reaction. Under these conditions, LexA is treated as the substrate and RecA* is the enzyme. If the K_M varies across different LexA proteins this would suggest that difference in *E. coli* RecA* mediated cleavage is largely due to the

interaction between *E. coli* RecA* and the different LexA proteins. However, if the K_M were similar this would suggest that the differences observed is due to the *intrinsic* cleavage rate of each LexA protein. When these data are analyzed in concert with direct binding data that reflect the K_D, we will also be able to speculate as to whether the RecA* binding step is the rate limiting step in catalysis of self-cleavage.

The aim of this study was to systematically characterize the self-cleavage properties of LexA proteins across different bacterial species. Our results demonstrate that the ability of LexA to self-cleavage in alkaline pH, is not a conserved feature of all LexA proteins, despite being a defining feature of *E. coli* LexA. However, RecA* aids in overcoming this lack of intrinsic self-cleavage observed with alkaline pH. Our work provides the initial foundation to start characterizing LexA proteins from more diverse bacterial species and to begin asking specific questions regarding molecular interaction between LexA and RecA*. Importantly, our results reveal that LexA proteins have different cleavage rates thus suggesting that these differences in cleavage rates may provide another layer of regulation to the SOS response across different bacteria species.

4.5 Material and Methods

4.5.1 LexA protein alignment. Amino acid sequences of LexA from different species was obtained from NCBI and aligned in BLAST. Crystal structure was downloaded from RCSB PDB database and modified using pymol.

4.5.2 Generation, expression, and purification of LexA proteins. Synthetic genes encoding *C. difficile, P. aeruginosa, B. subtilis, M. tuberculosis,* and *V. cholerae* LexA were obtained from Integrated DNA Technologies and cloned into a pET41 expression vector with a N-terminal poly-His tag and a PKA phosphorylation site. The *E. coli*

construct had been previously generated in the lab and was used in the protein purification steps. The enzymes were heterologously expressed in *E. coli,* BLR(DE3) expression cell lines followed by a one-step purification using the N-terminal tag. Briefly, expression cells were grown to an OD_{595} of 0.4-0.6 at which point each protein was induced by adding 1 mM IPTG and after 3 hr of growth, cells were harvested, and the pellet was stored at -80 ^{o}C. Cell pellets were resuspended and lysed using BugBuster Mastermix (EMD-Millipore). Lysates were centrifuged for 20 min at 10,000 rpm. Clarified lysates were affinity purified with HisPur resin (Thermo Fisher) using 300 mM imidazole to elute. Purified LexA was dialyzed at 4 ^{o}C into a buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, and 10% glycerol. The purified products were predominantly full-length, and the purity of each protein was determined by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and Coomassie staining. Protein concentrations were determined using Bradford standard curve method and stored at -80 ^{o}C

4.5.3 Alkali-mediated cleavage assays of LexA proteins. To determine the pH profile of each LexA protein, purified proteins were mixed in a 1:1 ratio with 2x cleavage buffer (100 mM Tris-Glycine-CAPS and 300 mM NaCl) with a pH ranging from 6.6-11.3 Reaction mixtures were incubated at 37 ^{o}C for 3 h. The cleavage reaction was quenched by adding 2x BME-containing Laemmli buffer and boiling at 95 ^{o}C for 10 min. The LexA cleavage reaction was visualized by running samples on a 15% SDS-PAGE gel and staining with Coomassie. The gels were imaged on a Typhoon Imager using res laser excitation at 633 nm with no filters. The fraction of cleaved proteins was calculated by dividing the density of the LexA cleavage products by the sum of the density of the LexA full-length and cleavage products using Quantity One (Bio-Rad). Each pH titrations were

performed in duplicate. The pKa was determined using the equation for a simple one proton catalysis:

$$f = \frac{((fraction\ cleavaed)\max x\ Ka)}{(Ka + [H+])}$$

For the quantitative kinetic analysis, 30 μL of purified protein was mixed with 30μL of 2x cleavage buffer (pH 10.6) and the mixture was incubated at 37 ^{o}C. At the specific time points, 5 μL of the reaction mixture was removed and the reaction was quenched with 2x BME-containing Laemmli buffer. The extent of cleavage was visualized over time on a 15% SDS-PAGE gel, stained with Coomassie, visualized on a Typhoon Imager and quantified as described previously. For each time point, the fraction of full-length LexA was calculated as described above and plotted versus time in Prism. Rate plots were fit to a first-order exponential equation $A = A_o e^{-kt}$. Prism was used to calculate half-life and k, the observed rate of cleavage. Kinetic experiments were done in duplicate.

4.5.4 RecA*-mediated cleavage of LexA proteins. RecA of *E. coli* was purified previously in the lab by a former graduate student, Zachary Hostetler as previously described (Hostetler, 2018). To activate RecA the following method was used: 10 μM of *E. coli* RecA was mixed with 1 mM ATPγS, 10 μM ssDNA and 1x-RecA activation buffer (25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT). The reaction was incubated for 1 h at 30 ^{o}C.

RecA*-mediated reaction was initiated by adding each LexA protein to activated RecA* to a final concentration of 2.5 μM of LexA and 5 μM of RecA*. The reaction was incubated at 37 ^{o}C and at specific time points an aliquot was removed, quenched with 2x

BME-containing Laemmli buffer, and boiled at 95 ^{0}C. The reactions were analyzed and quantified as described above.

Chapter 5: Future Directions and Concluding Remarks

Future directions and concluding remarks

The studies presented here are broad in scope and provide insight into all aspects of the SOS response. Collectively these studies shed light on the molecular mechanisms regulating the SOS response and the functional consequences of SOS activation. The motivation behind this work was twofold. First, we wanted to expand upon the canonical studies of the SOS response and understand the function of this stress response pathway in the context of a complex physiologic environment. Second, we wanted to address the conservation of the SOS response across bacterial species by probing the molecular dynamics of the key SOS regulator, LexA.

In Chapter 2, we began by taking a global look at the SOS response. Here, we asked whether the SOS response is necessary for *E. coli* to colonize the murine gastrointestinal tract. Prior to this study, the role of the SOS response in modulating commensal *E. coli* survival in a complex diverse environment such as the gastrointestinal tract was unknown. The results in Chapter 2 demonstrate that a functional SOS response is critical for an *E. coli* commensal to successfully colonize its natural habitat, the mammalian gut. This suggests that environmental conditions in the gut result in genotoxic stress that, in turn, favors a functional SOS response. Our results further address whether perturbing the host or the microbial flora impacts the requirement of an SOS response. In our study, we found that elimination of the endogenous gut microbiota abrogates the requirement of a functional SOS response for successful *E. coli* colonization. This work contributes to a growing body of literature that highlights the importance of the SOS response for bacteria to successfully interact with the surrounding environment. This work begins to describe environments that are

relevant inducers of the SOS response and specifically, the sources of genotoxic stress in these environments. Lastly, this work has important implications for bacterial evolution in the gut in the absence of exogenous stressors, such as antibiotics. While prior literature has focused on how antibiotics may activate stress response pathways to accelerate genomic adaptations, our work suggests that these same processes may be active in a healthy gut microbiome and thus driving genomic plasticity in the absence of antibiotics (Andersson, 2014; Gibson, 2014). While this study enhanced our understanding of the importance of the SOS response in the context of a natural environment, additional questions remain. These questions will be addressed in depth later but are briefly laid out here: First, future studies should be aimed at probing specific effector proteins that are responsible for the reduced fitness of the SOS-off *E. coli* strain. This would provide more insight into alternative functions of the SOS response outside of DNA damage repair that may be important for bacterial survival and proliferation. Second, future work should aim to directly test the relationship between the SOS response and genomic changes in the context of a healthy gut microbiome so we can better understand factors that increase bacteria evolvability.

In Chapter 3, we began to narrow our focus by studying one specific effector protein of the SOS response, colicin. In this chapter, we explored the function of colicin production for a commensal *E. coli* strain in the context of an unperturbed, complex environment, a healthy mammalian gut. Prior to this study, the fitness benefit of colicin production was examined in either theoretical models or largely synthetic environments. Further, little work had been done investigating the role of colicin production for a commensal *E. coli* isolate. The results in Chapter 3 support the hypothesis that in a well-mixed environment, such as a culture tube, colicins are important for competition with other colicin-sensitive *Enterobacteriaceae*. However, our results demonstrate that colicin

production is not required for an *E. coli* commensal to successfully colonize the mammalian gut. This finding suggests that for a commensal colonizing its primary environment, stressful events that may favor colicin production are not routinely encountered. The results of this chapter call into question the role of colicins for persistence in the gastrointestinal tract and begin to inform us which environments necessitate colicin production. The results of this chapter undoubtably contribute to our understanding of colicin biology but leave us with some pressing unanswered questions. Future studies will be discussed later in detail but are summarized here: First, we should address whether colicin production is required during direct competition for an environmental niche, and, second, we should examine whether fluctuations to the gastrointestinal tract require colicin production for bacterial survival.

In Chapter 4, we narrowed our focus even further and studied the molecular dynamics of LexA self-cleavage reaction. Prior to this study, the biochemical properties of LexA across different species had been underexplored and lacked systematic evaluation. We were interested in whether the self-cleavage activity of LexA was conserved across phylogenetically-diverse bacterial species. The results presented in Chapter 4 highlight variability in alkaline-induced LexA self-cleavage activity across different bacterial species. Work in Chapter 4 began to address the conservation of the LexA:RecA* interaction by focusing on how these different LexA proteins cleave in the presence of *E. coli* RecA*. This study represents initial work that takes a novel comparative biology approach towards understanding the molecular mechanisms behind SOS activation. Chapter 4 is a work-in-progress, but preliminary work done in this chapter sets the foundation for more rigorous investigation into the molecular interactions between LexA and RecA*.

The work presented in the first two chapters of this thesis provides the groundwork for further evaluating the functional role of the SOS response and its effector proteins in complex, dynamic environments. The work summarized in the last chapter provides an initial framework for dissecting the molecular interface between LexA and RecA. In the next sections I will address future directions that aim to either solve unanswered questions or build upon results presented in the associated chapter.

5.1 SOS response and colonization

5.1.1 Systematic evaluation of SOS effector proteins important for *E. coli* colonization.

Our results in Chapter 2 reveal that the SOS response is critical for *E. coli* to robustly colonize the gastrointestinal tract. Our approach relied on global inactivation of the SOS response by preventing the self-cleavage reaction of LexA, thus reducing the expression of all the genes in the SOS regulon. In this study we did not explore whether it was the general global inactivation of the SOS response responsible for the colonization defect, or whether it was a direct result of reduced expression of specific SOS regulated genes. Systematically investigating the consequences of perturbing SOS-regulated genes could yield some very interesting and possibly surprising results.

In the SOS literature it is clear that the SOS regulon is more diverse than a simple DNA repair pathway as proteins involved in virulence, persister formation, and bacterial warfare can all be regulated by the SOS response (Zhang, 2000; Gillor, 2008; Galhardo *et al.*, 2009; Dorr, 2010; Li., 2010; Bielaszewska, 2012; Bernier, 2013). Further, the SOS regulon in *E. coli* consists of ~40 genes and some of these genes have no known function (Simmons, 2008). For example, several toxin-like genes are induced during SOS response, but the physiologic impacts have yet to be deciphered

(Magnuson, 2007; Unoson, 2008). It is possible that the inability to repair genotoxic stress causes the colonization defect, but it is also possible one of these “non-canonical” aspects of the SOS response is responsible for the colonization defect. It is also conceivable that the colonization defect is a result of a combination of multiple genes in the SOS regulon being repressed. Systematically assessing specific components of the SOS response that are required for colonization would further define the contribution of the SOS response to bacterial survival in the mammalian gut and would provide additional insight into the molecular requirements for colonization.

MP1 is an excellent model organism to further examine this question as it is genetically tractable, and its genome encodes genes that may be important for colonization, some of which are SOS regulated in other bacteria (Bisognano, 2004). Presently, the SOS regulon of MP1 is unknown, but this could be addressed by inducing the SOS response *in vitro* and comparing the transcriptional responses of the wild-type strain and the SOS-off strain (Courcelle, 2001; Cirz, 2006, 2007; Walter, 2014). After making systematic deletions via recombineering, we will repeat our murine colonization experiment with these genetic backgrounds that have an otherwise intact SOS response. We expect that those genes which are specifically important for colonization will have a defect similar to the SOS-off strain.

5.1.2 Evaluating SOS-induced mutagenesis during colonization of the mammalian gut.

Our results in Chapter 2 demonstrate that an *E. coli* commensal that has a constitutively-off SOS response cannot robustly colonize the gut of a healthy mouse. This result suggests that during colonization of their primary habitat, gut commensals experience genotoxic stress and the SOS response is required for full-fitness. Activation

of the SOS response in the presence of DNA damage, results in a transient hypermutator phenotype due to the up-regulation of error-prone translesional polymerases (Kubiak., 2017). Inactivation of the SOS response prevents the associated hypermutator phenotype (Mo, 2016). With these results in mind, we postulate whether the inability to become a transient hypermutator contributes to the colonization defect of the SOS-off strain.

There are suggestions in the literature that adaptive mutations are critical for successful bacterial colonization of various host sites. In the gastrointestinal tract, in a seminal paper by Giraud, et. al., they observed two key phenomena: one, bacterial mutation rates increase during mouse colonization of the gastrointestinal tract; two, there is a significant disadvantage to being constitutively mutagenic (Giraud, 2001). In a follow-up paper, the authors demonstrate how an adaptive mutation contributes to successful bacterial colonization of the murine gut (Giraud, 2008). In the upper respiratory tract, recent genomic sequencing of *S. aureus* suggest that commensal strains acquire de novo mutations during 'routine' colonization, yet the mechanisms governing genomic adaptation in commensals have not been elucidated (Young, 2017). The results in Chapter 2 allow us to theorize that the SOS response could be contributing to the increase in adaptive mutations of a commensal during colonization.

Our data in Chapter 2 suggest that the SOS response is active in the murine gut, however, we did not test whether this induced a transient hypermutator state that could contribute to subsequent adaptive mutations. The models established in Chapter 2 enable us to investigate SOS-induced mutagenesis in both a healthy, unperturbed gut and in an inflammatory gut. To begin, we can assess genomic mutations in MP1 during colonization of a healthy gut. We can solo colonize the murine gut with either the wild-type strain or the SOS-off strain. Then we can perform periodic whole-genome

sequencing of each strain as it persists in the gut. If the SOS response contributes to adaptive mutations, we would expect to see a greater degree of genetic changes in the wild-type strain relative to the SOS-off strain. That said, it is possible we will be unable to detect genomic mutations in either strain. This result could either be because most mutations are deleterious and thus undetectable, or the selective pressures in the gut do not promote bacterial adaptation in the same way other locations in the host might.

Building on the studies in an unperturbed gut, one can imagine perturbing the gut environment and assessing whether SOS-induced mutagenesis drives genomic adaptation in the setting of a fluctuating environment. To start, we can assess SOS-induced mutagenesis during gut inflammation. It is possible that an inflammatory environment requires faster genomic adaptation for continued bacterial survival in this new environment jam-packed with new-fangled environmental insults. To test this idea, we can repeat the experimental set-up detailed in the previous section but in the setting of DSS-induced colitis. Overall, the results from these investigations will inform us whether the SOS response contributes to bacterial evolvability in the gut.

5.1.3 The SOS response contribution to *E. coli* survival and evolvability during antibiotic therapy

The model developed in Chapter 2 provides a natural gut environment in which to study the role of the SOS response in the setting of antibiotic therapy. The detrimental effects of antibiotic therapy on the gut microbiome is well established (Becattini, 2016). However, the molecular pathways bacteria utilize to survive and adapt to these exogenous threats are largely unknown. Using our solo colonization model and our SOS-off strain, we could address whether the SOS response is critical for survival and the acquisition of antibiotic resistance in the gastrointestinal tract during antibiotic

treatment. A previous genetic study demonstrated that inactivation of LexA decreased survival and acquired antibiotic resistance in an *E. coli* murine thigh infection model (Cirz, 2006). Additionally, deleting the SOS-regulated error-prone polymerase in *Mycobacterium tuberculosis* attenuated virulence and abrogated antibiotic resistance in a mouse infection model (Boshoff, 2003). The results of those studies suggest that the SOS response is critical for the acquisition of antibiotic resistance and, significantly, preventing SOS induction could reduce survival and acquired antibiotic resistance. Despite these formative studies, whether this pattern holds true in additional clinically relevant models is largely underexplored

To address this question, we can envision two experimental set-ups. One set-up would involve a short-course of antibiotic treatment (3 days) which would model a pulse dose of antibiotic therapy. The other set-up would involve an extended course of sub-therapeutic antibiotic treatment, which would model chronic antibiotic therapy. In both experimental set-ups, we can assess bacterial survival during and after antibiotic treatment as well as each strain's resistance profiles. Further, to assess the overall changes in the genomic landscape of each strain, subsequent whole-genome sequence can be performed. Investigating the SOS response in the context of antibiotic therapy will provide more insight into the molecular mechanisms that drive bacterial survival and the acquisition of antibiotic resistance in the gut. Additionally, if inactivation of the SOS response prevents acquired antibiotic resistance in this setting, this result would argue strongly for further developing therapeutics aimed at targeting the SOS response to impede bacterial evolution.

5.1.4 Does modulating the SOS response impact *E. coli* colonization and evolvability in the gut?

The results of Chapter 2 highlight the importance of SOS inactivation but do not address whether modulating the activity of the SOS response can alter the fitness of *E. coli* during colonization of the gastrointestinal tract. Previous work in our lab demonstrated that LexA self-cleavage rates can be altered through amino acid substitutions in the cleavage domain such that we can generate LexA variants that are hypo-cleavers and hyper-cleavers (Mo, 2016). Strikingly, when *E. coli* strains were generated containing these LexA variants in the place of wild-type LexA, we saw a gradation of SOS activation as a function of LexA cleavage rate. Using these strains in a well-defined laboratory setting, we probed the effects of an altered LexA self-cleavage rate on fitness and mutation rate of the bacteria. In the presence of DNA damaging stress, the strains harboring LexA cleavage variants had reduced fitness relative to the wild-type strain. Further, stress-induced mutagenesis was accelerated in the strain containing a hyper-cleavable LexA. From this study we concluded that LexA activity may have been evolved an intermediate phenotype in order to effectively balance all functions of the SOS response. While the results of this study are compelling, this work was completed *in vitro* in defined culture conditions. Thus, it remains unclear whether different levels of SOS induction could influence bacterial fitness and survival in a complex natural environment. Moving these variants into MP1 and repeating the colonization experiments would allow us to directly test our previous conclusion in a physiologic system. This work would provide insight into the dynamic mechanisms by which evolve in order to bacteria survive and adapt in the context of a complex diverse environment.

5.2 Colicin production in the gastrointestinal tract

5.2.1 Examination of colicin production during *in vivo* competition with colicin-sensitive strain.

In Chapter 3 we demonstrated that during colonization of the gastrointestinal tract, a colicin-deficient MP1 strain can colonize with equal efficiency as a wild-type MP1 strain. In our model, the colicin-deficient strain remained resistant to the colicin because only the colicin gene was disrupted during construction of this mutant, leaving the immunity gene intact. This allowed us to test specifically the fitness benefit associated with colicin production. From this result we concluded that *E. coli* living in its primary habitat may not encounter situations in which colicin production is essential. Further, we demonstrated that a colicin-resistant and colicin-producing strain can co-exist with equal abundance in a natural habitat. However, we did not test how this interaction changes in the presence of a colicin-sensitive strain competing for the same environmental niche. Our *in vitro* results suggest that colicin production will enable MP1 to outcompete a colicin-sensitive strain, but this might not be the case in a natural setting. Reports in the colicin literature comparing *in vitro* conditions with the natural environment suggest that *in vitro* results may not predict what will happen in a complex natural environment. Specifically, researchers found that in a well-mixed culture *in vitro,* colicin-producing strains cannot coexist with colicin-sensitive or -resistant strains, yet these phenotypes are all recovered in a natural population (Gordon, 1998). In Chapter 3 the colicin-resistant strain persisted with equal numbers as the wild-type colicin producing strain, suggesting our model accurately reflects what is recovered in a natural population. It is unclear to what extent a colicin-sensitive strain can co-exist with a colicin-producing strain in an unperturbed environmental model. Expanding our findings with a colicin-

sensitive strain would significantly advance our understanding of the natural role of colicins in mediating competition dynamics in a complex microbial community. Presently, we have made an MP1 strain that lacks both the colicin gene and the immunity gene (Manuela Roggiani from the Goulian lab). We plan to repeat the mouse colonization experiments with this strain in competition with the wild-type colicin-producing strain. It is possible that the colicin-sensitive strain will be rapidly out-competed. This result would suggest that the two strains are directly competing for space and resources in the gut and colicin production provides a competitive advantage by eliminating the sensitive strain. It is also possible that the colicin-sensitive strain will persist in the gut but with decreased abundance relative to the colicin-producing strain. This result would suggest a fitness benefit to colicin production, but the associated cost of colicin production (lysis of the producing cell) enables colicin-sensitive strains to persist. It is also conceivable that there will be no competition defect and both strains will colonize with equal fitness. This result would continue to support our previous finding that in a natural unperturbed niche, colicin-mediated competition is absent. Conversely, it could also mean that the two strains are not localized to the same site in the gut. If the sensitive strain outcompetes the wild-type strain, this would be surprising, but would suggest that colicin production is ultimately too costly for the producing bacteria. Clearly, any outcome of this experiment would provide new insight into the fitness benefits associated with colicin production.

5.2.2 Investigating environmental perturbations and colicin production.

Colicins are incredibly prevalent throughout prokaryotes and as such must be serving some function for bacteria existing within microbial communities (Klaenhammer, 1988; Gordon, 1998; Riley, 1998; Riley, 2000). Yet, questions remain regarding what

ecological environments require colicin production and promote colicin-dependent microbial competition. Building on this question, it is known that colicin production is modulated by stress, but it is unclear what environments provide relevant stressors that promote colicin production. From the results in Chapter 3, we can begin to define what environments may induce colicin production. We speculate that when a commensal is inhabiting its primary environment, colicin production may not be required for survival and if there is microbial competition, the outcome does not depend on colicin production. That said, it remains unclear whether colicin production is critical for a commensal to survive when its natural habitat is perturbed. Our MP1 model and our colicin-deficient strain are uniquely positioned to explore whether perturbations to the environment necessitate colicin production. We hypothesize that colicin production is critical for a commensal during fluctuations of the environment such as changes in the diet, host immune status, or microbial flora. We can systematically address these different scenarios and test whether the colonization capacity of the colicin-deficient strain is altered. This work would not only contribute to the field of colicin biology but would provide insight into the types of microbial interactions that are ongoing during perturbations of the mammalian gut. The results of this study would also build upon results in Chapter 2 where we explored the role of the SOS response in a complex physiologic environment. Colicin production and the SOS response are intimately linked. Environments that require colicin production for successful bacteria persistence and survival would also suggest a role for the SOS response. Thus, using our SOS-off mutant we could explicitly test this hypothesis.

5.2.3 Does commensal colicin production influences microbial diversity in the murine gut?

In Chapter 3 we addressed the impact of deleting MP1's colicin on the fitness of MP1 during gut colonization, but we did not investigate whether perturbing colicin production altered the surrounding microbiota. Within the colicin literature it is hypothesized that colicin production mediates strain as well as community diversity in complex environments (Cascales, 2007). Further, the underlying mechanisms that act to maintain a healthy gut microbiome are largely unknown. The microbial abundance and complexity of the mammalian gastrointestinal tract is a prime environment in which colicins could impact the composition of the surrounding microbiome. However, only a few studies have assessed the *in vivo* effect of bacteriocin, colicins produced by non-*E. coli* species, in a healthy gut microbiome. Significantly, the few studies that been published demonstrate that the microbiota is transformed by the addition of colicins. In one study, administration of a bacteriocin producing *Lactobacillus salivarius* strain altered the composition of the gut microbiota in both pigs and mice and the microbial change was dependent on bacteriocin production (Riboulet-bisson, 2012). In a comparative study, the effects on the microbiota of five different bacteriocins were examined and in general, the relative abundances of the bacteria phyla were not affected by the presence of bacteriocin producers, but there were significant changes in the family and genus level (Umu, 2016). Additionally, bacteriocin, Bac-21, from *Enterococcus faecalis,* perturbed competing enterococcal strains and significantly decreased the abundance of the Gram-negative family Deferribacteraceae (Kommineni, 2015). Interestingly, in all these studies, the presence of bacteriocins impacted the broader microbial community, not just the predicted sensitive strains. None of these studies, however, addressed alterations to the microbiota when a colicin-producing

strain is removed from a healthy gut microbiome. We can utilize our MP1 model to specifically ask whether the gut microbiome is affected by the elimination of a colicin-producing commensal. To understand whether MP1 colicin impacts the general microbial community at large, we could employ 16S rRNA high throughput pyrosequencing to assess the gut community of mice colonized with either the wild-type MP1 strain or the colicin-deficient MP1 strain. The results of this study would provide insight into bacterial associated effectors that could mediate the composition of the gut microbiota.

5.3 Defining the molecular interaction of LexA from different species with RecA*.

Although the LexA protein is widely conserved, prior to this thesis, it remained unclear what aspects of the critical LexA self-cleavage reaction are conserved across bacterial species. In Chapter 4, we demonstrated the LexA self-cleavage rates in the presence of alkaline pH can vary extensively, with some LexA proteins not cleaving at all under these conditions. Conversely, *E. coli* RecA*-mediated cleavage induced the LexA self-cleavage reaction for all LexA proteins tested, albeit with different rates. In that chapter, we briefly discussed ongoing efforts to parse out whether differences in RecA*-mediated cleavage were due to altered intrinsic rates of LexA self-cleavage or whether they are due to differing affinities of each LexA protein for *E. coli* RecA*. We proposed utilizing radiolabeled LexA to evaluate initial cleavage rates of each LexA protein in the presence of limiting amounts of *E. coli* RecA* in order to calculate the kinetic K_M and k_{cat} parameters for RecA*-mediated cleavage. Determining K_M for each LexA:RecA* pair would provide an important step towards resolving how *E. coli* RecA* is interacting with each LexA protein. If the K_M values are similar, this would suggest that the catalytic steps involving LexA self-cleavage are the predominant drivers of species-associated

differences observed in the presence of *E. coli* RecA*. This provocative result would suggest that the molecular interaction between LexA and RecA* remained conserved across evolution and it is the self-cleavage property of LexA that evolved to have different rates of self-cleavage. If the K_M values are different, this would suggest that the interaction with *E. coli* RecA* is altering the rate of the cleavage reaction in pre-catalytic steps or potentially in post-catalytic product dissociation steps. Preliminary data with $LexA_{Tb}$ suggest the K_M is different compared to established literature values for $LexA_{Ec}$, but this assay needs to be optimized and repeated before any conclusions can be made.

Deciphering the K_M of each LexA protein for *E. coli* RecA* is an important first step towards developing a molecular model of the LexA:RecA* interactions. However, defining this property only captures one aspect of the molecular interactions between LexA and RecA*. A schematic of the molecular steps for the LexA:RecA interaction is described in Figure 17. The K_M value has the potential to reflect all the steps of the RecA*-mediated LexA cleavage reaction. It will be critical to also determine the LexA:RecA* binding equilibrium (K_D) using catalytically inactive LexA variants. Defining both properties will provide insight as to whether it is the binding of LexA with RecA* or whether it is an additional step in the reaction that accounts for the differing RecA*-mediated cleavage rates. If the K_D and K_M values are similar to one another and differ across LexA constructs, this could be strongly suggestive of a simple binding model in which RecA captures LexA in a cleavable conformation upon binding. If instead, K_D values are similar, but K_M values differ, this result would suggest the possibility of a post-binding step, such as a conformational change in the formed complex, that impacts the overall rate of cleavage. Determining these kinetic constants for each of the different LexA proteins with *E. coli* RecA* will thus help to define the LexA:RecA* reaction and will

provide the necessary framework to begin probing molecular residues that are critical to the LexA:RecA* interaction.

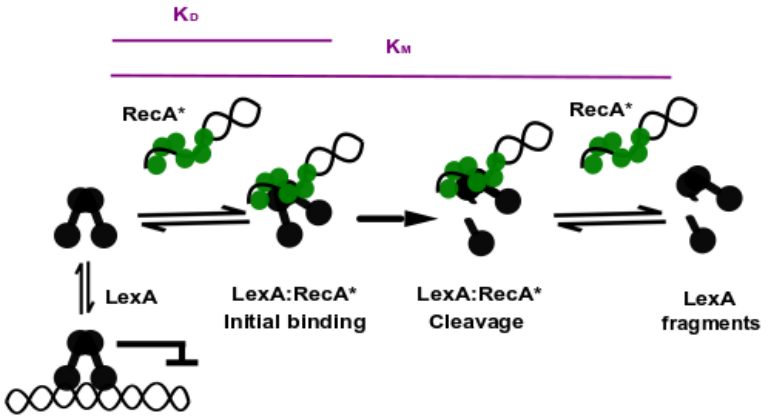


Figure 17: Expanded LexA:RecA* Reaction Model. Proposed mechanistic steps of the RecA*-mediated LexA self-cleavage. The K_D reflects the initial LexA:RecA* binding equilibrium. The K_M reflects all steps in the cleavage reaction starting from full-length LexA to the LexA fragments.

To probe the initial binding equilibrium (K_D) of each LexA protein with RecA*, we can employ a fluorescence spectroscopic method using acridone, a fluorescent unnatural amino acid, as a minimally-perturbing and sensitive site-specific probe that can specifically report on LexA binding to RecA* (Speight *et al.*, 2013). This method has previously been established in our lab by a former graduate student, Zachary Hostetler. When acridone-labeled LexA binds to RecA* there is a shift in fluorescence intensity or fluorescence anisotropy. Utilizing a stopped-flow apparatus, we can measure a time-dependent and concentration-dependent change in fluorescence anisotropy, which allows us to obtain quantitative kinetic information with respect to LexA binding to RecA* (Hostetler, unpublished). Using a competitive binding assay previously established by Zachary Hostetler, we can determine K_D values for each LexA protein. In this assay,

unlabeled catalytically-inactive LexA proteins from different species would be pre-incubated with RecA*, allowing an equilibrium to be established (Figure 18).

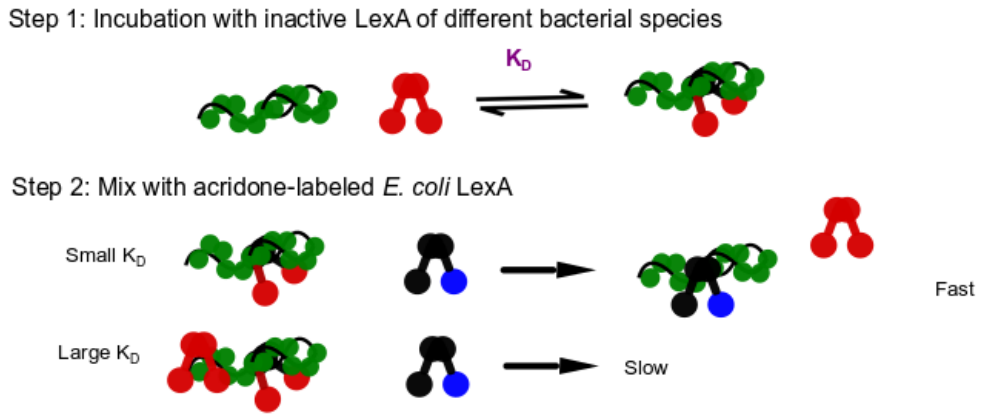


Figure 18: **Experimental set-up with acridone**. This experimental set-up is designed to measure the strength of competitive binding with *E. coli* RecA* between LexA of different species (red cartoon LexA) and acridone-labeled *E. coli* LexA (black and blue cartoon, where the blue indicates the acridone label).

Then we would rapidly mix in acridone-labeled $LexA_{Ec}$, monitor changes in anisotropy, and calculate the initial rate of binding. Repeating this analysis with different concentrations of unlabeled catalytically-inactive LexA from different species will allow us to monitor the anisotropy change and calculate the initial binding rate at different concentrations of unlabeled LexA thus generating a binding curve where the K_D can be determined. Comparing the binding curves of each LexA protein to the curve for $LexA_{Ec}$ would reveal differences in binding affinity.

In addition to the above studies focused on *E. coli* RecA, expanding both the radiolabeled LexA and the acridone-based binding assays to RecA proteins from multiple species could further elucidate how the LexA:RecA* interface may have evolved across the phylogenetic tree. Ideally, we would include RecA proteins from Gram-positive and mycobacterial species. Gathering this information would allow us to make two formative contributions to the SOS field. One, it would be the first determination of

the kinetic parameters surrounding the complex interaction between LexA and RecA* for another bacterial species. For example, characterizing $LexA_{Tb}$ and $RecA^*_{Tb}$ may provide clues as to how the SOS regulon is regulated *in vivo* and would inform rational inhibitor design. Two, it would allow us to make informed conclusions regarding how the LexA:RecA* interface may have evolved across the phylogenetic tree. Understanding how protein-protein interfaces evolve across the phylogenetic tree can provide insight into the essential features that must be maintained, and those that are "tunable", adapting the pathway to best suit the needs of a particular species. For example, it is possible in this scenario a LexA protein from a Gram-positive species would cleave more rapidly in the presence of a RecA* protein from a Gram-positive species then it did in the presence of *E. coli* RecA*, which could suggest co-evolution of this interface. Alternatively, the converse may also be true in that a LexA proteins across species would show the same spectrum of cleavage rates with a Gram-positive LexA. This result would be more suggestive of the fact that LexA intrinsic self-cleavage has adapted independent of the RecA* interface.

5.4 Concluding remarks

This thesis explores uncharted territory in the 60+ years of study into the SOS response. Work in this thesis expands upon the traditional biochemical approach commonly utilized in the lab to address biologically-significant questions. Work in Chapters 2 and 3 exploit a mouse model that mimics the natural environment in order to address important questions that were unanswered in both the SOS literature and the colicin literature. However, the conclusions in each of these chapters barely scratch the surface of what there is to understand regarding the functional role of the SOS response and its effector proteins in natural habitats. Additionally, our work only investigated the

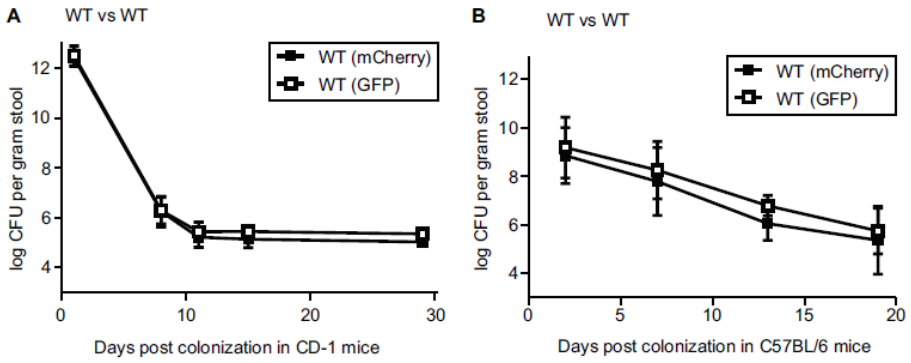
SOS response in *E. coli.* The SOS regulon is highly variable across different bacterial species, thus it is important to extend this work to other clinically-relevant bacterial pathogens. We hope that work done in this thesis will inspire new questions aimed at understanding how this critical stress response pathway permits bacteria to successfully interact, adapt, and survive within their surrounding environment.

Further work done in Chapters 2 and 3 re-emphasizes the importance of using a physiologically relevant model in order to address biological questions. Specifically, in Chapter 3 our results were different from previous reports in the literature. We postulate these differences were largely due to the utilization of a streptomycin-treated mouse model in prior studies, which eliminates a significant portion of the microbial diversity. It is possible, our results more accurately reflect the natural environment because our studies employ a mouse-specific *E. coli* isolate and eliminate the need for sustained antibiotic treatment during mouse colonization experiments.

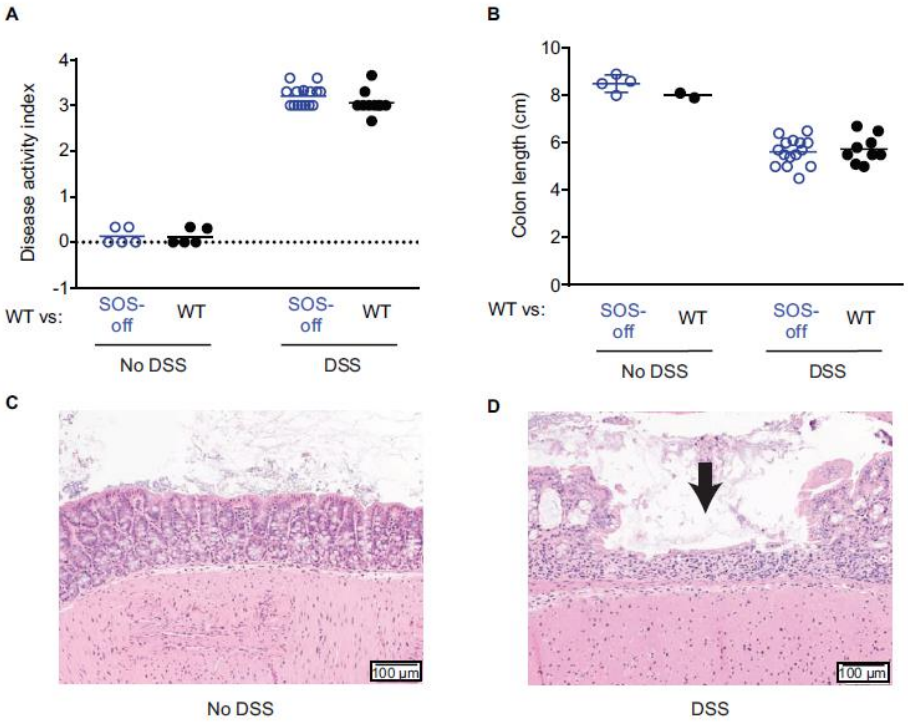
In Chapter 4 we returned to biochemistry and addressed LexA self-cleavage rates across different bacterial species. While we focused on biochemically characterizing the differences in LexA self-cleavage activity across bacterial species, our discovery raises the interesting question of *why* these self-cleavage rates are different. We hope highlighting these differences will lead to further investigation into whether SOS regulation across different bacterial species is tuned to reflect the characteristics of each unique bacterial species. Taken together, the three data chapters presented in this thesis support the hypothesis that the SOS response is far more complex than a simple DNA repair pathway and is critical component for successful bacterial interactions with the surrounding ominous environment.

Appendix

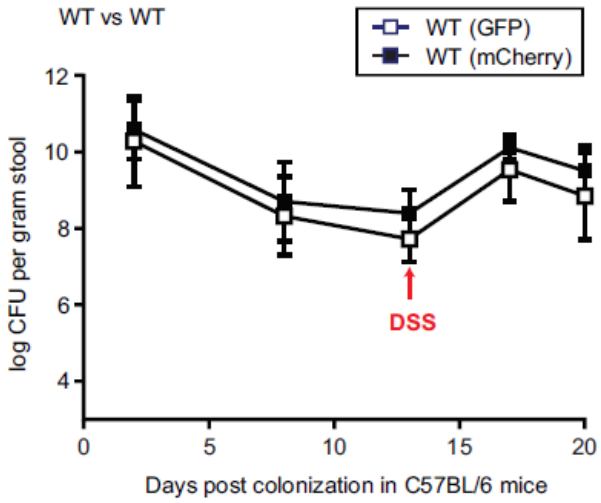
Supporting information for Chapter 2



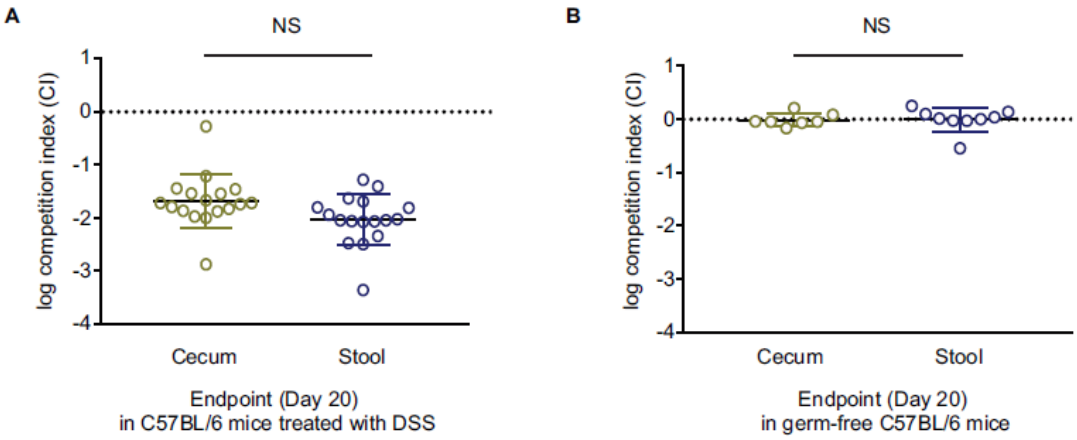
Supplemental Figure 1: Shown are the log CFU per gram of stool for WT:WT control competition experiments in either CD-1 or C57BL/6 mice. Each symbol represents the mean of all individual mice with the standard deviation shown. The limit of detection was 10^2 for the CD-1 mice and 10^3 for C57BL/6 mice.



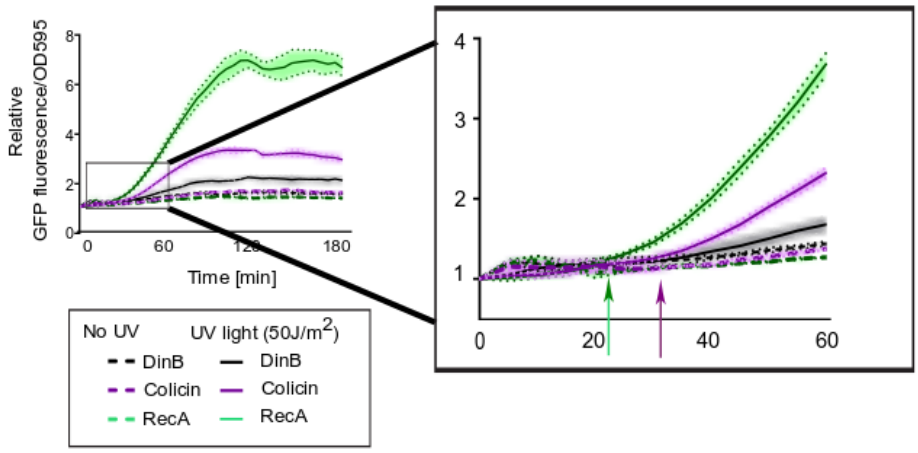
Supplemental Figure 2: Clinical evidence of DSS-induced inflammation. (A) Disease activity index was determined at the time of euthanasia. The index combines and averages scores of weight loss, stool consistency and bleeding. (B) Colon length of DSS-treated versus mice that did not receive DSS. Colon length was measured shortly after euthanasia. Representative histological images of a colon from (C) a mouse not receiving DSS and (D) a mouse receiving DSS. Arrow indicates inflammatory infiltrate in the colonic mucosa and a region of ulceration.



Supplemental Figure 3: Shown are the log CFU per gram of stool for WT:WT DSS-treated control competition experiments with C57BL/6 mice. Each symbol represents the mean of all individual mice with the standard deviation shown. The limit of detection was 10^3.



Supplemental Figure 4: Comparison of cecal versus stool results for WT:SOS-off competition in the DSS-treated (A) or germ-free experiments (B). At the time of euthanasia cecal contents were collected and prepared similar to the feces. Shown are the competition index calculations. Each circle represents an individual mouse. The comparison between the cecal and stool samples was made using a two-tailed unpaired Student *t* test. NS, no statistical significance (p>0.01).



Supplemental Figure 5: SOS reporter assay with *recA* promoter. The promoter activity of *recA* is compared to the promoter activity of *colicin* and *dinB*. GFP induction for *recA* promoter or *colicin* promoter is indicated by the purple and green arrows, respectively. GFP induction for the *recA* promoter begins around 20-25 min and 30-35 min for the *colicin* promoter. The time of increased promoter activity was determined based on statistically significant increase in GFP fluorescence relative to baseline as determined by two-tailed unpaired student *t* test.

Bibliography

Adikesavan, A. K., Katonis, P., Marciano, D. C., Lua, R., Herman, C., Lichtarge, O. (2011) Separation of recombination and SOS response in Escherichia coli RecA suggests LexA interaction sites, *PLoS Genetics*, 7(9): e100224.

Ambur, O. H., Davidesn, T., Frye, S. A., Balasingham, S.V., Lagesen, K., Rognes, T., Tonjum, T. (2009) Genome dynamics in major bacterial pathogen', *FEMS Microbiology Reviews*, 33(3), pp. 453.

Andersson, D. I., Hughes, D. (2014) Microbiological effects of sublethal levels of antibiotics., *Nature reviews. Microbiology*. Nature Publishing Group, 12(7), pp. 465.

Antunes, L. C. M, Han, J., Ferreira, B. R., Lolic, P., Borchers, C. H., Finlay, B. B . (2011) Effect of Antibiotic Treatment on the Intestinal Metabolome, *Antimicrobial Agents and Chemotherapy*, 55(4), pp. 1494.

Baharoglu, Z., Bikard, D., Mazel, D. (2010) Conjugative DNA transfer induces the bacterial SOS response and promotes antibiotic resistance development through integron activation', *PLoS Genetics*, 6(10), pp. 1–10.

Baharoglu, Z., Krin, E., Mazel, D. (2012) Connecting environment and genome plasticity in the characterization of transformation-induced SOS regulation and carbon catabolite control of the Vibrio cholerae integron integrase, *Journal of Bacteriology*, 194(7), pp. 1659.

Baharoglu, Z., Mazel, D. (2011) Vibrio cholerae Triggers SOS and Mutagenesis in Response to a Wide Range of Antibiotics: a Route towards Multiresistance, *Antimicrobial Agents and Chemotherapy*, 55(5), pp. 2438.

Baharoglu, Z., Mazel, D. (2014) SOS, the formidable strategy of bacteria against aggressions, *FEMS Microbiology Reviews*, 38, pp. 1125..

Battesti, A., Majdalani, N., Gottesman, S. (2011) The RpoS-Mediated General Stress Response in Escherichia coli, *Annual Rev Microbiology*, 65, pp. 189.

Bazett, M., Bergeron, M., Haston, C. K. (2016) Streptomycin treatment alters the intestinal microbiome, pulmonary T cell profile and airway hyperresponsiveness in a cystic fibrosis mouse model, *Science Reports*, 6(19189), pp. 1–13.

Be'er, A., Zhang, H. P., Florin, E.-L., Payne, S. M., Ben-Jacob, E., Swinney, H.L. (2009) Deadly competition between sibling bacterial colonies, *PNAS*, 106(2), pp. 428.

Beaber, J. W., Hochhut, B., Waldor, M. K. (2004) SOS response promotes horizontal dissemination of antibiotic resistance genes., *Nature*, 427(6969), pp. 72.

Becattini, S., Taur, Y., Pamer, E. G. (2016) Antibiotic-Induced Changes in the Intestinal Microbiota and Disease, *Trends in Mol. Med*, 22(6), pp. 458.

Beck, P. L*., Xavier, R.,* Wong, J., Ezedi, I., Mashimo, H., Mizoguchi, A., Mizoguchi, E., Bhan, A. K., Podolsky, D. K. (2004) Paradoxical roles of different nitric oxide synthase isoforms in colonic injury, *Am J Physiol Gastrointest Liver Phyisol*, 286, pp. 137.

Begley, M., Hill, C. (2015) Stress Adaptation in Foodborne Pathogens, *Annual Rev. Food Sci. Technol*, 6, pp. 191.

Bell, J. C., Kowalczykowski, S. C. (2016) RecA : Regulation and Mechanism of a Molecular Search Engine, *Trends in Biochemical Sciences*, 41(6), pp. 491.

Bernier, S. P., Lebeaux, D., DeFranscesco, A.S., Valomon, A., Soubigou, G., Coppee, J-Y, Ghigo, J-M., Beloin, C. (2013) Starvation, Together with the SOS Response, Mediates High Biofilm-Specific Tolerance to the Fluoroquinolone Ofloxacin, *PLoS Genetics*, 9(1).

Bernstein, C., Bernstein, H., Payne, C. M, Beard S. E., Schneider, J*.* (1999) Bile Salt Activation of Stress Response Promoters in Escherichia coli, *Current Microbiology*, 39, pp. 68..

Bielaszewska, M., Idelevich, E.A., Zhang, W., Bauwens, A., Schaumburg, F., Mellmann, A., Peters, G., Karch, H*.* (2012) Effects of Antibiotics on Shiga Toxin 2 Production and Bacteriophage Induction by Epidemic Escherichia coli O104: H4 Strain, *Antimicrobial Agents and Chemotherapy*, 56(6), pp. 3277.

Bisognano, C. Kelley, W. L., Estoppey, T., Francois, P.,…..Vaudaux, P*.* (2004) A RecA-LexA-dependent Pathway Mediates Ciprofloxacin-induced Fibronectin Binding in Staphylococcus aureus, *Journal of Biological Chemistry*, 279(10), pp. 9064.

Boshoff, H. I. M., Reed, M. B., Barry, C. E., Mizrahi, V*.* (2003) DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in Mycobacterium tuberculosis, *Cell*, 113(2), pp. 183.

Bunnell, B. E., Escobar, J. F, Bair, K. L., Sutton, M. D., Crane, J. K. (2017) Zinc blocks SOS-induced antibiotic resistance via inhibition of RecA in Escherichia coli, *PLoS ONE*, 12(5), pp. 1–20.

Butala, M., Sonjak, S., Kamensek, S., Hodoscek, M., Browning, D.F., Bertok-Zgur, D., Busby, J. W. (2012) Double locking of an Escherichia coli promoter by two repressors prevents premature colicin expression and cell lysis, *Molecular Medicine Reports*, 86(1), pp. 129.

Butala, M., Žgur-Bertok, D., Busby, S. J. W. (2009) The bacterial LexA transcriptional repressor, *Cellular and Molecular Life Sciences*, 66(1), pp. 82.

Cascales, E., Buchanan, S. K., Duche, D., Kleanthous, C., Lloubes, R., Postle, K., Riley, M., Slatin, S., Cavard, D. (2007) Colicin Biology, *Microbiology and Molecular Biology Reviews*, 71(1), pp. 158.

Chandran, A. V., Srikalaivani, R., Paul, A., Vijayan, M. (2019) Biochemical characterization of Mycobacterium tuberculosis LexA and structural studies of its C-terminal segment research papers, *Acta Cryst*. International Union of Crystallography, pp. 1–15.

Chen, Z., Yang, H., Pavletich, N. P. (2008) Mechanism of homologous recombination from the RecA – ssDNA / dsDNA structures, *Nature.* 45, pp.489.

Cirz, R. T., Chin, J. K., Andes, D. R., Crecy-Lagard, V., Craig, W. A., Romesberg, F.,E. (2005) Inhibition of mutation and combating the evolution of antibiotic resistance, *PLoS Biology*, 3(6), pp. 1024.

Cirz, R. T., O'Neill, B. M., Hammond, J., A., Head, S. R., Romesberg, F. E. (2006) Defining the Pseudomonas aeruginosa SOS response and its role in the global response to the antibiotic ciprofloxacin, *Journal of Bacteriology*, 188(20), pp. 7101.

Cirz, R. T. Jones. M., Gingles, N., Minogue, T., Peterson, S. N., Romesber, F. E. (2007) Complete and SOS-mediated response of Staphylococcus aureus to the antibiotic ciprofloxacin, *Journal of Bacteriology*, 189(2), pp. 531.

Cirz, R. T., Romesberg, F. E. (2006) Induction and inhibition of ciprofloxacin resistance-conferring mutations in hypermutator bacteria, *Antimicrobial Agents and Chemotherapy*, 50(1), pp. 220.

Claverys, J., Håvarstein, L. S. (2007) Cannibalism and fratricide : mechanisms and raisons d ' être', *Nature Reviews Microbiology*,5, pp. 219..

Conway, T., Krogfelt, K. A., Cohen, P. S. (2004) The Life of Commensal Escherichia coli in the Mammalian Intestine, *EcoSal Plus*, 8.3.1.2, pp. 1–16.

Cooper, H., Murthy, S., Shah, R., Sedergran, D. (1993) Clinicopathologic study of dextran sulfate sodium experimental murine colitis, *Laboratory investigation; a journal of technical methods and pathology*, 69(2), p. 238.

Cornforth, D. M., Foster, K. R. (2013) Competition sensing : the social side of bacterial stress responses', *Nature Publishing Group*. Nature Publishing Group, 11(4), pp. 285.

Courcelle, J., Khodursky, A., Peter, B., Brown, P. O., Hanawalt, P. C. (2001) Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient Escherichia coli, *Genetics*, 158(1), pp. 41.

Cox, M. M. (2007) Regulation of bacterial RecA protein function, *Critical reviews in biochemistry and molecular biology*, 42(1), pp. 41.

Culyba, M. J., Kubiak, J.M., Mo, C. Y., Goulian, M., Kohli, R. M. (2018) Non-equilibrium repressor binding kinetics link DNA damage dose to transcriptional timing within the SOS gene network, *PLOS Genetics*, 14(6), pp e100740.

Culyba, M. J., Mo, C. Y., Kohli, R. M. (2015) Targets for Combating the Evolution of Acquired Antibiotic Resistance, *Biochemistry*, 54, pp. 3573.

Czaran, T. L., Hoekstra, R. F., Paige, L. (2001) 'Chemical warfare between microbes', *PNAS*, 99(2), pp. 786.

Czyz, A., Wrobel, B., Wegrzyn, G. (2000) Vibrio harveyi bioluminescence plays a role in stimulation of DNA repair, *Microbiology*, (146), pp. 283..

Diard, M., Bakkeren, E., ……Hardt, W-D. (2017) Inflammation boosts bacteriophage transfer between Salmonella spp., *Science*, 355, pp. 1211.

Dong, T. G., Dong, S., Catalona, C., Moore, R., Liang, X., Mekalanos, J. J. (2015) Generation of reactive oxygen species by lethal attacks from competing microbes, *PNAS,* 112(27), pp. 2181.

Dorr, T., Lewis, K., Vulic, M. (2009) SOS Response Induces Persistence to Fluoroquinolones in Escherichia coli, *PLoS Genetics*, 5(12), pp. e1000760.

Dorr, T., Vulic, M., Lewis, K. (2010) Ciprofloxacin Causes Persister Formation by Inducing the TisB toxin in Escherichia coli, *PLoS Biology*, 8(2), pp. 29.

Dri, A., Moreau, P. L. (1994) Control of the LexA regulon by pH: evidence for a reversible inactivation of the LexA repressor during the growth cycle of Escherichia coli, *Molecular Microbiology*, 12(4), pp. 621.

Durrett, R., Levin, S. (1997) Allelopathy in Spatially Distributed Populations, *Journal of Theoretical Biology*, 185, pp. 165.

Easton, A. M., Kushner, S. R. (1983) Transcription of the uvrD gene of Escherichia coli is controlled by the lexA repressor and by attenuation, *Nucleic acids research*, 11(24), pp. 8625.

Eppinger, M., Mammel, M., K., Leclerc, J. E., Ravel, J., Cebula, T. A. (2011) Genomic anatomy of Escherichia coli O157: H7 outbreaks, *PNAS*, 108(50), pp. 20142.

Erill, I., Campoy, S., Barbé, J. (2007) Aeons of distress: An evolutionary perspective on the bacterial SOS response, *FEMS Microbiology Reviews*, 31(6), pp. 637.

Fang, F. C., Frawley, E. R., Tapscott, T., Vazquez-Torres, A. (2017) Bacterial Stress Responses during Host Infection, *Cell Host and Microbe*, 20(2), pp. 133.

Foster, J. W. (1999) When protons attack: microbial strategies of acid adaptation, *Current Opinion in Microbiology*, 2, pp. 170.

Foster, P. L. (2005) Stress responses and genetic variation in bacteria, *Mutation Research*, 569(6), pp. 3.

Foster, P. L. (2007) Stress-Induced Mutagenesis in Bacteria, *Crit Rev Biochem Mol Biol*, 42, pp. 373.

Friedberg, E. C., Wagner, R., Radman, M. (2002) Specialized DNA Polymerases, Cellular Survival, and the Genesis of Mutations, *Science*, 296, pp. 1627.

Friedman, N., Vardi, S., Ronen, M., Alon, U., Stavans, J. (2005) Precise Temporal Modulation in the Response of the SOS DNA Repair Network in Individual Bacteria, *PLoS Biology*, 3(7), pp. e238.

Galhardo, R. S., Do, R., Yamada, M., Friedberg, E.C., Hasting, P. J., Nohmi, T., Rosenberg, S. M. (2009) DinB upregulation is the sole role of the SOS response in stress-induced mutagenesis in Escherichia coli, *Genetics*, 182(1), pp. 55.

Galhardo, R. S., Hastings, P. J., Rosenberg, S. M., (2007) Mutation as a Stress Response and the Regulation of Evolvability, *Crit Rev Biochem Mol Biol*, 42(5), pp. 399.

Gamage, S. D., Patton, A. K., Strasser, J. E., Chalk, C.L., Weiss, A. A. (2006) Commensal Bacteria Influence Escherichia coli O157: H7 Persistence and Shiga Toxin Production in the Mouse Intestine, *Infection and Immunity*, 74(3), pp. 1977.

Garner, C. D., Antonopoulos, D. A., Wagner, B., Duhamel, G. E., Keresztes, I., Ross, D. A., Young, V. B., Altier, C., (2009) Perturbation of the Small Intestine Microbial Ecology by Streptomycin Alters Pathology in a Salmonella enterica Serovar Typhimurium Murine Model of Infection, *Infection and Immunity* 77(7), pp. 2691.

Ghazaryan, L., Tonoyan, L., Ashhab, A. A., Soares, M., Gillor, O. (2014) The role of stress in colicin regulation, *Arch Microbiol*, 196, pp. 753.

Gibson, M. K., Pesesky, M. W., Dantas, G., (2014) The Yin and Yang of Bacterial Resilience in the Human Gut Microbiota, *Journal of Molecular Biology*. 426(23), pp. 3866.

Giese, K. C., Michalowski, C. B., Little, J. W., (2008) RecA-Dependent Cleavage of LexA Dimers, *Journal of Molecular Biology*, 377(1), pp. 148.

Gillings, M. R. (2014) Integrons: past, present, and future, *Microbiology and Molecular Biology Reviews*, 78(2), pp. 257.

Gillor, O., Giladi, I., Riley, M. A. (2009) Persistence of colicinogenic Escherichia coli in the mouse gastrointestinal tract, *BMC Microbiology*, 9, pp. 165.

Gillor, O., Vriezen, J. A., Riley, M. A. (2008) The role of SOS boxes in enteric bacteriocin regulation, *Microbiology*, 154(6), pp. 1783.

Giraud, A., Matic, V., Tenaillon, O., Clara, A., Radman, M., Fons, M., Taddei, F. (2001) Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut, *Science*, 291(5513), pp. 2606.

Giraud, A., Arous, S.,…..Cerf-Bensussan, N. (2008) Dissecting the genetic components of adaptation of Escherichia coli to the mouse gut, *PLoS Genetics*, 4(1), pp. 0052.

Goneau, L., Hannan, T. J., MacPhee, R. A., Schwartz, D. J., Macklaim, J. M., Gloor, G. B., Razvi, H., Reid, G., Hulgren, S. J., Burton, J. B. (2015) Subinhibitory Antibiotic Therapy Alters Recurrent Urinary Tract Infection Pathogenesis through modulation of bacterial virulence and host immunity, *mBio*, 6(2), pp. e00356..

Gordon, D. M., O'Brien, C. (2006) Bacteriocin diversity of the frequency of multiple bacteriocin production in Escherichia coli, *Microbiology*, 152(11), pp. 3239.

Gordon, D. M., Riley, M. A., Pinou, T. (1998) Temporal changes in the frequency of colicinogeny in Escherichia coli from house mice, *Microbiology*, (144), pp. 2233..

Goswami, K., Chen, C., Xiaoli, L., Eaton, K., A., Dudley, E.,G. (2015) Coculture of Escherichia coli O157: H7 with a Nonpathogenic E. coli Strain Increases Toxin Production and Virulence in a Germfree Mouse, *Infection and Immunity*, 83(11), pp. 4185.

Gotoh, H. Kasaraneni N., Devineni, N., Dallo, S. F., Weitao, T. (2010) SOS involvement in stress-inducible biofilm formation, *Biofouling*, 26(5), pp. 603.

Grozdanov, L., Raasch, C., Schulze, J., Sonnenborn, U., Gottschalk, G., Hacker, J., Dobrindt, U. (2004) Analysis of the Genome Structure of the Nonpathogenic Probiotic Escherichia coli Strain Nissle 1917, *Journal of Bacteriology*, 186(16), pp. 5432..

Guerin, E., Cambray, G., Alberola-Sanchez, N., Campoy, S., Erill, I., De Ra, S., Gonzalez-Zorn, B., Barbe, J., Ploy, M-C., Mazel, D. (2009) 'The SOS response controls integron recombination.', *Science*, 324(5930), pp. 1034.

Hentges, D., Pongpech, P., Que, J. (1990) Hypothesis : How Streptomycin Treatment Compromises Colonisation Resistance Against Enteric Pathogens in Mice, *Microbial Ecology in Health and Disease*, 3(3), pp. 105.

Herschman, H. R., Donald, A. (1967) Comparative Study of the Events Associated with Colicin Induction, *J. Bacteriology,* 94(3), pp. 691.

Hibbing, M. E., Fuqua, C., Parsek, M. R., Peterson, S. B. (2010) Bacterial competition: surviving and thriving in a microbial jungle, *Nature Reviews Microbiology*, 8(13), pp. 15.

Hocquet, D., Llanes, C., Thouverez, M., Kulasekara, H., D., Bertrand, X., Plesait, P., Mazel, D., Miller, S. I. (2012) Evidence for induction of integron-based antibiotic resistance by the SOS response in a clinical setting', *PLoS Pathogens*, 8(6), pp. e1002778.

Hostetler, Z. M., Ferrie, J. J., Bornstein, M. R., Sungwienwong, I., Petersson, E. J., Kohli, R. M. (2018) Systematic Evaluation of Soluble Protein Expression Using a Fluorescent Unnatural Amino Acid Reveals No Reliable Predictors of Tolerability, *ACS Chemical Biology*, 13, pp. 2855.

Hughes, E. R., Winter, M.G.,…….Winter, S.E. (2017) Microbial Respiration and Formate Oxidation as Metabolic Signatures of Inflammation-Associated Dysbiosis, *Cell Host and Microbe*, 21, pp. 208.

Iguchi, A., Thomson, N. R.,…..Frankel, G. (2009) Complete Genome Sequence and Comparative Genome Analysis of Enteropathogenic Escherichia coli O127: H6 Strain E2348 / 69, *Journal of Bacteriology*, 191(1), pp. 347.

Imlay, J. A., Chin, S. M., Linn, S. (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro, *Science*, 240(4852), pp. 640.

Jarvik, T., Smillie, C., Groisman, E. A., Ochman, H. (2010) Short-Term Signatures of Evolutionary Change in the Salmonella enterica Serovar Typhimurium 14028 Genome, *Journal of Bacteriology*, 192(2), pp. 560.

Jena, N. (2012) DNA damage by reactive species : Mechanisms, Mutation and Repair, *J. Biosci*, 37(3), pp. 503.

Jerman, B., Butala, M., Zgur-Bertok, D. (2005) Sublethal Concentrations of Ciprofloxacin Induce Bacteriocin Synthesis in Escherichia coli, *Antimicrobial Agents and Chemotherapy*, 49(7), pp. 3087..

Joelsson, A., Liu, Z., Zhu, J. (2006) Genetic and Phenotypic Diversity of Quorum-Sensing Systems in Clinical and Environmental Isolates of Vibrio cholerae, *Infection and Immunity*, 74(2), pp. 1141.

Johansson, M. E. V., Gustafsson, J., K., Sjoberg, K.E., Petersson, J., Holm, L., Sjovall,

H., Hansson, G.C. (2010) Bacteria Penetrate the Inner Mucus Layer before Inflammation in the Dextran Sulfate Colitis Model, *PLoS ONE*, 5(8), pp. e12238.

Justice, S. S., Hunstad, P., Seed, P. C., Hultrgren, S. J. (2006) Filamentation by Escherichia coli subverts innate defenses during urinary tract infection, *PNAS*, 103(52), pp. 19884.

Kerr, B., Riley, M. A., Feldman, M., W., Bohannan, B. J. M. (2002) Local dispersal promotes biodiversity in a real-life game of rock – paper – scissors, *Nature*, 418(1998), pp. 2865.

Kimsey, H. H., Waldor, M. K. (2009) Vibrio cholerae LexA Coordinates CTX Prophage Gene Expression, *Journal of Bacteriology*, 191(22), pp. 6788.

Kirkup, B. C., Riley, M. A. (2004) Antibiotic-mediated antagonism leads to a bacterial game of rock – paper – scissors in vivo, *Nature*, 428, pp. 694.

Kisker, C., Kuper, J. and Van Houten, B. (2013) Prokaryotic Nucleotide Excision Repair, *Cold Spring Harbor Perspectives in Biology*, pp. 1–18.

Klaenhammer, T. R. (1988) Bacteriocins of lactic acid bacteria, *Biochimie*, 70(11171), pp. 337.

Kommineni, S., Bretl, D., J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., Cao, Y., Bousounis, P., Kristich, C. J., Salzman, N. H. (2015) Bacteriocin production augments niche competition by enterococci in the mammalian GI tract, *Nature*, 526(7575), pp. 719.

Kovačič, L., Paulic, N., Leonardi, A., Hodnik, V., Anderluh, G., Podlesek, Z., Zgur-Bertok, D., Krizaj, I., Butala, M. (2013) Structural insight into LexA-RecA* interaction, *Nucleic Acids Research*, 41(21), pp. 9901.

Krin, E., Cambray, G., Mazel, D. (2014) The superintegron integrase and the cassette promoters are co-regulated in Vibrio cholerae, *PLoS ONE*, 9(3), pp. e91194.

Kubiak, J., M., Culyba, M., J., Lui, M., Y., Mo, C., Y., Goulian, M., Kohli, R. M. (2017) A Small-molecule inducible synthetic circuit for control of the SOS gene network without DNA damage, *ACS Synthetic Biology*, 6, pp. 2067.

Lasaro, M., Liu, Z., Bisher, R., Kelly, K., Chattopadhyay, S., Paul, S., Sokurenko, E., Zhu, J., Goulian, M. (2014) Escherichia coli isolate for studying colonization of the mouse intestine and its application to two-component signaling knockouts, *Journal of Bacteriology*, 196(9), pp. 1723.

Lasaro, M. A., Salinger, N., Zhang, J., Wang, Y., Zhong, Z., Goulian, M., Zhu, J. (2009) F1C Fimbriae Play an Important Role in Biofilm Formation and Intestinal Colonization by the Escherichia coli Commensal, *Applied and Environmental Microbiology*, 75(1), pp. 246.

Lenski, R, E., Rose, M., Simpson, S., Tadler, S. (1991) Long-term experimental evolution in E. coli, *American Naturalist*, 138(6), pp. 1315.

Li, B., Smith, P., Horvath, D. J., Romesberg, F. L., Justice, S. S. (2010) SOS regulatory elements are essential for UPEC pathogenesis, *Microbes and Infection*, 12 (8) pp. 662.

Little, J. W. (1984) Autodigestion of lexA and phage lambda repressors, *PNAS*, 81(5), pp. 1375.

Lloubes, R., Lazdunski, C., Granger-Schnarr, M., Schnarr, M. (1993) DNA sequence determinants of LexA-induced DNA bending, 21(10), pp. 2363.

Love, P. E., Yasbin, R. E. (1986) Induction of the Bacillus subtilis SOS-like response by Escherichia coli RecA protein, *PNAS*, 83, pp. 5204.

Lozupone, C., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., Knight, R. (2012) Diversity, stability and resilience of the human gut microbiota, *Nature*, 489(7415), pp. 220..

Luo, C., Walk, S. T., Gordon, D. M., Feldgargen, M., Tiedje, J. M., Konstantinidis, K., T. (2011) Genome sequencing of environmental Escherichia coli expands understanding of the ecology and speciation of the model bacterial species, *PNAS*, 108(17), pp. 7200.

Luo, Y., Pfuetzner, R. A., Mosimann, S., Paetzel, M., Frey, E. A., Cherney, M., Kim, B., Little, J. W., Strynadka, N. C. J. (2001) Crystal structure of LexA: A conformational switch for regulation of self-cleavage, *Cell*, 106(5), pp. 585.

Magnuson, R. D. (2007) Hypothetical Functions of Toxin-Antitoxin Systems, *Journal of Antimicrobial Chemotherapy*, 189(17), pp. 6089.

Maiques, E., Ubeda, C., Campoy, S., Salvador, N., Lasa, I., Novick, R. P., Barbe, J., Penades, J. R. (2006) 'β -Lactam Antibiotics Induce the SOS Response and Horizontal Transfer of Virulence Factors in Staphylococcus aureus, *Journal of Bacteriology,* 188(7), pp. 2726.

Majeed, H., Lampert, A., Ghazaryan, L., Gillor, O. (2013) The Weak Shall Inherit: Bacteriocin-Mediated Interactions in Bacterial Populations, *PLoS ONE*, 8(5), pp. e63837.

Malachowa, N., Deleo, F. R. (2010) Mobile genetic elements of Staphylococcus aureus, *Cellular and Molecular Life Sciences*, 67(18), pp. 3057.

Matsushiro, A., Sato, K., Miyamoto, H., Yamamura, T., Honda, T. (1999) Induction of Prophages of Enterohemorrhagic Escherichia coli O157: H7 with Norfloxacin, *Journal of Bacteriology*, 181(7), pp. 2257.

Mcglynn, P., Savery, N. J., Dillingham, M. S. (2012) The conflict between DNA replication and transcription, *Molecular Microbiology*, 85(1), pp. 12.

Meador, J. P., Caldwell, M. E., Cohen, P. S., Conway, T. (2014) Escherichia coli Pathotypes Occupy Distinct Niches in the Mouse Intestine, *Infection and Immunity,* 82(5), pp. 1931.

Mellies, J. L., Haack, K. R., Galligan, D. C. (2007) SOS Regulation of the Type III Secretion System of Enteropathogenic Escherichia coli, 189(7), pp. 2863.

Mesak, L. R., Miao, V., Davies, J. (2008) Effects of subinhibitory concentrations of antibiotics on SOS and DNA repair gene expression in Staphylococcus aureus, *Antimicrobial Agents and Chemotherapy*, 52(9), pp. 3394.

Miller, C., Thomsen, L. E., Gaggero, C., Mosseri, R., Ingmer, H., Cohen, S. N. (2004) SOS response induction by beta-lactams and bacterial defense against antibiotic lethality, *Science*, 305(5690), pp. 1629.

Mo, C. Y., Manning, S. A., Roggiani, M., Culyba, M. J., Samuels, A. N., Sniegowski, P. D., Goulian, M., Kohli, R. M. (2016) Systematically Altering Bacterial SOS Activity under Stress Reveals Therapeutic Strategies for Potentiating, *mSphere*, 1(4), pp. e00163.

Mo, C. Y., Birdwell, L. D., Kohli, R. M. (2014) Specificity determinants for autoproteolysis of Lexa, a key regulator of bacterial SOS mutagenesis, *Biochemistry*, 53(19), pp. 3158.

Mobley, H. L., Green, D. H., Trifillis, A. L., Johnson, D. E., Chippendale, G. R., Lockatell, V. C., Jones, B. D., Warren, J. W. (1990) Pyelonephritogenic Escherichia coli and Killing of Cultured Human Renal Proximal Tubular Epithelial Cells : Role of Hemolysin in Some Strains, *Infection and Immunity*, 58(5), pp. 1281.

Mrak, P., Podlesek, Z., van Putten, J. P. M., Bertok-Zgur, D. (2007) Heterogeneity in expression of the Escherichia coli colicin K activity gene cka is controlled by the SOS system and stochastic factors, *Molecular Genetic Genomics*, 277, pp. 391.

Muhldorfer, I., Hacker, J., Keusch, G. T., Acheson, D. W., Tschape, H., Kane, A. V., Ritter, A., Olschlager, T., Donohur-Rolfe, A. (1996) 'Regulation of the Shiga-Like Toxin II Operon in Escherichia coli', *Infection and Immunity*, 64(2), pp. 495.

Murphy, K. C., Campellone, K. G. (2003) Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic E. coli., *BMC Molecular Biology*, 4, pp.11.

Mustard, J. A., Little, J. W. (2000) Analysis of Escherichia coli RecA Interactions with LexA , λ CI , and UmuD by Site-Directed Mutagenesis of recA, *Journal of Bacteriology* 182(6), pp. 1659.

Myhal, M., Laux, D., Cohen, P. (1982) Relative colonizing abilities of human fecal and K 12 strains of Escherichia coli in the large intestines of streptomycin-treated mice, *European Journal of Clinical Microbiology*, 1(3), pp. 186.

Nataro, J. P., Baldini, M., Kaper, J. B., Black, R. E., Bravo, N., Levine, M. M. (1985) Detection of an adherence factor of enteropathogenic Escherichia coli with a DNA probe, *Journal of Infectious Disease*, 152(3), pp. 560.

Nedialkova, L. P., Denzler, R., Koeppel, M. B., Diehl, M., Ring, D., Wille, T., Gerlack, R. G., Stecher, B. (2014) Inflammation Fuels Colicin Ib-Dependent Competition of Salmonella Serovar Typhimurium and E. coli in Enterobacterial Blooms, *PLoS Pathogens*, 10(1), pp. e1003844.

Ni, J., Shen, D. T-C., …….Wu, G. D. (2017) A role for bacterial urease in gut dysbiosis and Crohn's disease, *Science Translational Medicine*, 9, pp eaah6888.

Ni, M., Yang, L., Liu, X-L., Qi, O. (2008) Fluence-Response Dynamics of the UV-Induced SOS Response in Escherichia coli, *Current Microbiology*, 57, pp. 521.

Oda, Y. (1987) Induction of SOS responses in Escherichia coli by 5-fluorouracil, *Mutation Research*, 183(2), pp. 103.

Oliveira, R. C. De, Laval, J., Boiteux, S. (1986) Induction of SOS and adaptive responses by alkylating agents in Escherichia coli mutants deficient in 3-methyladenine-DNA glycosylase activities, *Mutation Research*, 183, pp. 11.

Ozkaya, O., Xavier, K. B., Dionisio, F., Balbontin, R. (2017) Maintenance of Microbial Cooperation Mediated by Public Goods in Single- and Multiple-Trait Scenarios, *Journal of Bacteriology*, 199(22), pp. e00297.

Paez, P. L., Becerra, M. C., Albesa, I. (2011) Comparison of Macromolecular Oxidation by Reactive Oxygen Species in Three Bacterial Genera Exposed to Different Antibiotics, *Cell Biochem Biophys*, 61, pp. 467.

Papavinasasundaram, K. G., Anderson, C., Brooks, P.C., Thomas, N. A., Movahedzadeh, F., Jenner, P. J., Colston, M. J., Davis, E. O. (2001) Slow induction of RecA by DNA damage in Mycobacterium tuberculosis, *Microbiology*, 147(12), pp. 3271.

Paul, S., Weaver-Million, S., Chattopadhyay, S., Sokurenjo, E., Merrikh, H. (2013) Accelerated gene evolution through replication-transcription conflicts, *Nature*, 495(7442), pp. 512.

Pennington, J. M., Rosenberg, S. M. (2007) Spontaneous DNA breakage in single living Escherichia coli cells., *Nature genetics*, 39(6), pp. 797.

Perse, M., Cerar, A. (2012) Dextran Sodium Sulphate Colitis Mouse Model : Traps and Tricks, *Journal of Biomedicine and Biotechnology*, 2012, pp. 1–13.

Pohlhaus Reineke, J., Kreuzer, K. J. (2005) Norfloxacin-induced DNA gyrase cleavage complexes block Escherichia coli replication forks, causing double-stranded breaks in vivo, *Molecular Microbiology*, 56(6), pp. 1416..

Quinones, M., Davis, B. M., Waldor, M. K. (2006) Activation of the Vibrio cholerae SOS response is not required for intestinal cholera toxin production or colonization, *Infection and Immunity*, 74, pp. 927.

Quinones, M., Kimsey, H. H., Waldor, M. K. (2005) LexA Cleavage Is Required for CTX Prophage Induction, *Molecular Cell*, 17, pp. 291.

Raffatellu, M. (2018) Learning from bacterial competition in the host to develop antimicrobials, *Nature Medicine*, 24, pp. 1097.

Rasko, D. A., Rosovitz, M. J.,…..Ravel, J. (2008) The Pangenome Structure of Escherichia coli : Comparative Genomic Analysis of E. coli Commensal and Pathogenic Isolates, *Journal of Bacteriology*, 190(20), pp. 6881.

Recacha, E., Machuca, J., de Alba, P. D., Ramos-Guelfo, M., Docobo-Perez, F., Rodriguez-Beltran, J., Blazuez, J., Pascual, A., Rodriguez-Martinez, J. M. (2017) Quinolone Resistance Reversion by Targeting the SOS Response, *mBio*, 8, pp.e00971.

Riboulet-bisson, E., Sturme, M. H. J.,……Ross, P. R. (2012) Effect of Lactobacillus salivarius Bacteriocin Abp118 on the Mouse and Pig Intestinal Microbiota, *PLoS ONE*, 7(2), p. e31143.

Riley, M. A. (1998) Molecular Mechanisms of Bacteriocin Evolution, *Annual Rev Genetics*, 32, pp. 255.

Riley, M. A., Cadavid, L., Collett, M. S., Neely, M. N., Adams, M. D., Phillips, C. M., Neel, J. V., Friedman, D. (2000) 'The newly characterized colicin Y provides evidence of positive selection in pore-former colicin diversification', *Microbiology*, 146, pp. 1671–1677.

Riley, M. A., Goldstone, C. M., Wertz, J. E., Gordon, D. (2003) A phylogenetic approach to assessing the targets of microbial warfare, *Journal of Evolutionary Biology*, 16, pp. 690.

Riley, M. A., Gordon, D. M. (1992) A survey of Col plasmids in natural isolates of Escherichia coli and an investigation into the stability of Col-plasmid lineages, *Journal of General Microbiology*, 138, pp. 1345.

Riley, M. A., Gordon, D. M. (1999) The ecological role of bacteriocins in bacterial competition, *Trends in Microbiology*, 7(3), pp. 129.

Riley, M. A., Wertz, J. E. (2002) BACTERIOCINS : Evolution, Ecology, and Application, *Annual Rev Microbiology*, 56, pp. 117.

Roland, K. L., Smith, M., H., Rupley, J. A., Little, J. W. (1992) In Vitro Analysis of Mutant LexA Proteins with an Increased Rate of Specific Cleavage, *J. Mol Biol*, 228, pp. 395.

Sale, J. E., Lehmann, A. R., Woodgate, R. (2013) Y-family DNA polymerases and their role in tolerance of cellular DNA damage, *Nature Review Mol Cell Biol*, 13(3), pp. 141.

Samuels, A. N., Roggiani, M., Zhu, J., Goulian, M., Kohli, R. M. (2019) The SOS response mediates sustained colonization of the mammalian gut, *Infection and immunity*, 87(2), pp. 1–15.

Schuldiner, S., Agmon, V., Brandsma, J., Cohen, A., Friedman, E., Padan, E. (1986) Induction of SOS Functions by Alkaline Intracellular pH in Escherichia coli, *Journal of Bacteriology*, 168(2), pp. 936

Sender, R., Fuchs, S., Milo, R. (2016) Revised Estimates for the Number of Human and Bacteria Cells in the Body, *PLoS Biology*, 14(8), pp. e1002533.

Sharma, A., Kottur, J., Narayanan, N., Nair, D. T. (2013) A strategically located serine residue is critical for the mutator activity of DNA polymerase IV from Escherichia coli, *Nucleic acids research*, 41(9), pp. 5104.

Simmons, L. A., Foti, J. J., Cohen, S. E., Walker, G. C. (2008) The SOS Regulatory Network, *EcoSal Plus*, 5.4.3, pp. 1–30.

Sinha, R., Hader, D. (2002) UV-induced DNA damage and repair: a review, *Photochem Photobiol Sci*, 1(4), pp. 224.

Siryaporn, A., Goulian, M. (2008) Cross-talk suppression between the CpxA – CpxR and EnvZ – OmpR two-component systems in E . coli, *Molecular Microbiology*, 70(2), pp. 494.

Slilaty, S. N., Little, J. W. (1987) 'Lysine-156 and serine-119 are required for LexA repressor cleavage: A possible mechanism, *PNAS*, 84, pp. 3987.

Smith, M. H., Cavenagh, M. M., Little, J. W. (1991) Mutant LexA proteins with an increased rate of in vivo cleavage, *PNAS*, 88(16), pp. 7356..

Smollett, K. L., Smith, K. M., Kahramanoglou, C., Arnvig, K. B., Buxton, R. S., Davis, E. O. (2012) Global analysis of the regulon of the transcriptional repressor LexA, a key component of SOS response in Mycobacterium tuberculosis, *Journal of Biological Chemistry*, 287(26), pp. 22004.

Sniegowski, P. D., Gerrish, P. J., Lenski, R. E. (1997) Evolution of high mutation rates in experimental populations of E. coli, *Nature*, 606, pp. 703..

Spees, A. M., Wangdi, T., Lopez, C. A., Kingsbury, D. D., Xavier, M. N., Winter, S. E., Tsolis, R. M., Baumler, A. J. (2013) Streptomycin-Induced Inflammation Enhances Escherichia coli Gut Colonization Through Nitrate Respiration, *mBio*, 4(4), pp. e00430.

Speight, L. C., Muthusamy, A. K., Goldberg, J. M., Warner, J. B., Wissner, R. F., Willi, T. S., Woodman, B. F., Mehl, R. A., Petersson, E. J. (2013) Efficient Synthesis and In vivo incorporation of Acridon-2-ylalanine, a Fluorescent Amino Acid for Lifetime and FRET/LRET Studies, *Journal American Chemical Society*, 135(50), pp. 18806.

Stalder, T., Barraud, O., Casellas, M., Dagot, C., Ploy, M-C. (2012) Integron involvement in environmental spread of antibiotic resistance, *Frontiers in Microbiology*, 3(119), pp. 1–14.

Stecher, B., Denzler, R.,…….Hardt, W-D. (2012) Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae, *PNAS*, 109(4), pp. 1269.

Stern, A. M., Hay, A. J., Liu, Z., Desland, F. A., Zhang, J., Zhong, Z., Zhu, J. (2012) The NorR Regulon Is Critical for Vibrio cholerae Resistance to Nitric Oxide and Sustained Colonization of the Intestines, *mBio*, 3(2), pp. e00013.

Stohl, E. A., Gruenig, M., C., Cox, M. M., Seifert, S. H. (2011) Purification and characterization of the RecA protein from Neisseria gonorrhoeae, *PLoS ONE*, 6(2), pp. e17101.

Streelman, A. J., Snyder, I. S, Six, E. W. (1970) Modifying Effect of Colicin on Experimental Shigella Keratoconjunctivitis, *Infection and Immunity*, 2(1), pp. 15.

Stubbendieck, R. M., Straight, D. (2016) Multifaceted Interfaces of Bacterial Competition, *Journal of Bacteriology*, 198(16), pp. 2145.

Sweeney, N. J., Laux, D. C., Cohen, P. S. (1996) Escherichia coli F-18 and E. coli K-12 eda Mutants Do Not Colonize the Streptomycin-Treated Mouse Large Intestine, *Infection and immunity*, 64(9), pp. 3504.

Taddei, F., Radman, M., Maynard-Smith, J., Toupance, B., Gouyon, P.H.,Godelle, B. (1997) Role of mutator alleles in adaptive evolution, *Nature*, 387, pp. 700.

Tompkins, J. D., Nelson, J. L., Hazel, J. C., Leugers, S. L., Stumpf, J. D., Foster, P. L. (2003) Error-prone polymerase, DNA polymerase IV, is responsible for transient hypermutation during adaptive mutation in Escherichia coli, *Journal of Bacteriology*, 185(11), pp. 3469..

Torsvik, V., Goksyr, J., Daae, F. L. (1990) High Diversity in DNA of Soil Bacteria, *Applied and Environmental Microbiology*, 56(3), pp. 782..

Toshima, H., Yoshimura, A., Arikawa, K., Hidaka, A., Ogasawara, J., Hase, A., Masaki, H., Nishikawa, Y. (2007) Enhancement of Shiga Toxin Production in Enterohemorrhagic Escherichia coli Serotype O157 : H7 by DNase Colicins, *Applied and Environmental Microbiology*, 73(23), pp. 7582.

Trastoy, R., Manso, T, Garcia-Fernandez, L., Blasco, L., Ambroa, A., Perez del Molino, M. L., Bou, G., Garcia-Contreras, R., Wood, T. K., Tomas, M. (2018) Mechanisms of Bacterial Tolerance and Persistence in the Gastrointestinal and Respiratory Environments, *Clinical Microbiology Reviews*, 31(4), pp. e00023.

Tyler, J. S., Beeri, K., Reynolds, J. L., Alteri, C. J., Skinner, K. G., Friedman, J. H., Eaton, K. A., Friedman, D. I. (2013) Prophage Induction Is Enhanced and Required for Renal Disease and Lethality in an EHEC Mouse Model, *PLoS Pathogens*, 9(3), pp. e1003236.

Úbeda, C., Maiques, E., Knecht, E., Lasa, I., Novick, R. P., Penades, J. R. (2005) Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci, *Molecular Microbiology*, 56(3), pp. 836.

Umu, O., Bauerl, C., Oostindjer, M., Pope, P., B., Hernandez, P. E., Perez-Martinez, G., Diep, D. B. (2016) The Potential of Class II Bacteriocins to Modify Gut Microbiota to Improve Host Health, *PLoS ONE*, 11(10), pp. e0164036.

Unoson, C., Wagner, E. G. H. (2008) A small SOS-induced toxin is targeted against the inner membrane in Escherichia coli', *Molecular Microbiology*, 70(1), pp. 258.

Vankemmelbeke, M., Healy, B., Moore, G. R., Kleanthous, C., Penfold, C. N., James, R. (2005) Rapid Detection of Colicin E9-Induced DNA Damage Using Escherichia coli Cells Carrying SOS Promoter- lux Fusions, *Journal of Bacteriology*, 187(14), pp. 4900.

van der Veen, S., van Schalkwijk, S., Molenaar, D., de Vos, W. M., Abee, T., Wells-Bennik, M. H. J. (2010) The SOS response of Listeria monocytogenes is involved in

stress resistance and mutagenesis, *Microbiology*, 156(2), pp. 374.

Wadolkowski, E. A., Laux, D. C., Cohen, P. S. (1988) Colonization of the streptomycin-treated mouse large intestine by a human fecal Escherichia coli strain: Role of adhesion to mucosal receptors, *Infection and Immunity*, 56(5), pp. 1036.

Wagner, P. L., Neely, M. N., Zhang, X., Acheson, D. W. Waldor, M. K., *Friedman, D. I.* (2001) Role for a phage promoter in Shiga toxin 2 expression from a Pathogenic Escherichia coli strain, *Journal of Bacteriology*, 183(6), pp. 2081.

Wagner, P. L., Neely, M. N., Zhang, X., Acheson, D. W., Waldor, M. K., Friedman, D. I. (2001) Role for a Phage Promoter in Shiga Toxin 2 Expression from a Pathogenic Escherichia coli Strain, *Journal of Bacteriology*, 183(6), pp. 2081.

Walker, D., Rolfe, M., Thompson, A., Moore, G. R., James, R., Hinton, J. C. D., Kleanthous, C. (2004) Transcriptional Profiling of Colicin-Induced Cell Death of Escherichia coli MG1655 Identifies Potential Mechanisms by Which Bacteriocins Promote Bacterial Diversity, *Journal of Bacteriology,* 186(3), pp. 866.

Wall, D. (2016) Kin recognition in bacteria, *Annual Rev Microbiology*, 70, pp. 143.

Walter, B. M., Rupnik, M., Hodnik, V., Anderluh, G., Dupuy, B., Paulic, N., Zgur-Bertok, D., Butala, M. (2014) The LexA regulated genes of the Clostridium difficile, *BMC Microbiology*, 14, pp. 88.

Walter, B. M., Cartman, S. T., Minton, N. P., Butala, M., Rupnik, M. (2015) The SOS Response Master Regulator LexA Is Associated with Sporulation , Motility and Biofilm Formation in Clostridium difficile, *PLoS ONE*, 10(12), pp. e0144763.

Winter, S. E., Winter, M.G.,…..Baumler, A. J. (2013) Host-Derived Nitrate Boosts Growth of E. coli in the Inflamed Gut, *Science*, 339, pp. 708..

Winterbourn, C. C., Kettle, A. J., Hampton, M. B. (2016) Reactive Oxygen Species and Neutrophil Function, *Annual Rev. Biochem*, 85, pp. 765.

Winterling, K. W., Levine, A. S., Yasbin, R. E., Woodgate, R. (1997) Characterization of DinR, the Bacillus subtilis SOS repressor, *Journal of Bacteriology*, 179(5), pp. 1698

Wloch-Salamon, D. M, Gerla, D., Hoekstra, R. F., de Visser, J. A. G. M. (2008) Effect of

dispersal and nutrient availability on the competitive ability of toxin-producing yeast, *Proceedings of the Royal Society B*, 275, pp. 535

Yan, Y., Kolachala, V., Dalmasso, G., Nguyen, H., Laroui, H., Sitaraman, S. V., Merlin, D. (2009) Temporal and Spatial Analysis of Clinical and Molecular Parameters in Dextran Sodium Sulfate Induced Colitis, *PLoS ONE*, 4(6), pp. e6073.

Young, B. C., Wu, C-H., …….Wilson, D. J. (2017) Severe infections emerge from commensal bacteria by adaptive evolution, *elife*, 6, p. e30637.

Yu, X., Egelman, E. H. (1993) The LexA Repressor Binds within the Deep Helical Groove of the Activated RecA Filament, *J. Mol Biol*, 231, pp. 29.

Zaslaver, A., Bren A., Ronen, M., Itzkovitz, S., Kikoin, I., Shavit, S., Liebermeister, W., Surette, M. G., Alon, U. (2006) A comprehensive library of fluorescent transcriptional reporters for Escherichia coli, *Nature Methods*, 3(8), pp. 623.

Zhang, A. P. P., Pigli, Y. Z., Rice, P. A., (2010) Structure of the LexA – DNA complex and implications for SOS box measurement, *Nature*, 466(7308), pp. 883.

Zhang, X., McDaniel, A. D., Wolf, L. E., Keusch, G. T., Waldor, M. K., Acheson, D. W. K. (2000) 'Quinolone Antibiotics Induce Shiga Toxin–Encoding Bacteriophages, Toxin Production, and Death in Mice', *The Journal of Infectious Diseases*, 181(2), pp. 664